

**THE ROLE OF IRON AND HAEM IN**  
**BREAST CANCER**

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## **ABSTRACT**

Iron is ubiquitous in the human body, fulfilling many crucial roles. However, accumulating evidence implicates excess iron as a carcinogen. This study aimed to further investigate the role of iron and haem in breast carcinogenesis and the potential utility of chelators in therapy.

We demonstrate that the transport machinery for iron and haem is dysregulated in breast cancer, with import being promoted and export down-regulated to allow the accumulation of intra-cellular iron. In vitro studies demonstrate that, in contrast to benign cells, malignant cells are capable of importing haem as well as ionic iron. Both iron and haem stimulate aggressive behaviour in malignant cells, up-regulating viability, proliferation, adhesion, migration and invasion. These changes are abrogated by iron chelation. In addition, the expression profile of iron and haem transporters is shown to favour intra-cellular accumulation even when iron is plentiful and import would be expected to be down-graded.

Overall this study suggests that breast cancer may be due to an inappropriate expression profile of iron and haem transporters, leading to excess intra-cellular iron which drives a malignant cell phenotype. In addition the action of chelators to downgrade malignant behaviour implies a potential therapeutic role.

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## ABBREVIATIONS

AMP	Adenosine monophosphate-activated protein
APC	Adenomatous polyposis coli
BC	Breast cancer
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein
BRCA1/2	Breast cancer 1/2
BrdU	5-bromo-2'-deoxyuridine
CDK	Cyclin-dependent kinase
CRC	Colorectal cancer
DAB+	3,3'-diaminobenzidine tetrachloride
DCIS	Ductal carcinoma in situ
DCytb	Duodenal cytochrome b
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
FCS	Fetal calf serum
FLVCR	Feline leukaemia virus subgroup C receptor
HCC	Hepatocellular carcinoma
HCP1	Haem carrier protein 1
HEPES	(N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HER	Human epithelial growth factor receptor
HIF1	Hypoxia inducible factor 1

HRE	Hypoxia responsive element
HRT	Hormone replacement therapy
IRE	Iron responsive element
IRP	Iron response protein
LCIS	Lobular carcinoma in situ
LRP1	LDL receptor related protein 1
MOPS	Morpholinepropanesulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZF-1	Myeloid zinc finger 1
NB	Normal breast
Nrf2	Nuclear factor erythroid 2-like 2
NTP	Nucleotide triphosphate
OCP	Oral contraceptive pill
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PR	Progesterone receptor
PVDF	Polyvinylidene fluoride
RCC	Renal cell carcinoma
RIPA	Radio-immunoprecipitation
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TBST	TBS with Tween
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TfR	Transferrin receptor

TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TNM	Tumour/nodes/metastases
UTR	Un-translated region

# **CHAPTER 1: INTRODUCTION AND BACKGROUND**

## **1.1 Breast Cancer**

### **1.1.1 Epidemiology**

According to the UK National Cancer Registries<sup>1-4</sup>, in 2006 breast cancer had the highest incidence of all malignancies in the UK. 45 500 new cases accounted for 31% of cancer diagnoses in women in 2006 (the next most common being lung cancer with 11%). Based on figures from 2001-2005, the lifetime risk of a woman in the UK developing breast cancer was calculated as 1 in 9 in February 2009<sup>5</sup>.

The disease is largely one of late middle and old age. 81% of cases occur in the over 50s, 48% in those aged between 50 and 69<sup>1-4</sup>.

The combination of high incidence and low mortality (greater than 80% five year survival) also means a high prevalence of breast cancer in the UK population. Based on data up to the end of 2004 it was estimated that 550 000 of the UK female population at the time had at some point been diagnosed<sup>6</sup>. This equates to 2% of the total UK female population, or almost 12% of women over 65. The incidence continues to rise; since the late 1970s there has been a 63% increase in the incidence of breast cancer in Britain (from 75 to 122 cases per 100 000).

Worldwide there are over 1 million new diagnoses of breast cancer made annually, comprising 10% of all new cancers and 23% of cancers in females<sup>7</sup>. 429 900 new cases are reported annually in Europe, 182 460 in North America<sup>8</sup>. The highest incidence was found in the USA, with a rate of over 100 cases per 100 000 females, whilst the lowest was in China, with less than 20<sup>7</sup>.

The advent of breast screening in the developed world saw a transient increase in the incidence of breast cancer as a pool of undiagnosed cases was brought to light. This occurred in the late 1980s in the UK<sup>1-3</sup>.

In the 1990s, the use of HRT (Hormone Replacement Therapy) to combat menopausal symptoms in middle-aged women contributed to increasing incidence of breast cancer<sup>9</sup>. However, this trend is now reversing within the 50-70 age-group, as increased awareness of the associated risks has decreased the uptake of HRT.

The lower rates previously seen in Eastern Europe and the Far East are now rising to approach those seen in the West<sup>10-12</sup>. This has been attributed to changes in reproductive behaviour, use of HRT, increasing obesity and increased consumption of alcohol.

### **1.1.2 Environmental Risk Factors**

#### *1.1.2.1 Socio-Economic Status*

Increased affluence confers a higher risk of developing breast cancer<sup>13-16</sup>. This likely reflects altered exposure to environmental factors as a consequence of differing lifestyles, education, and career plans and expectations. Interestingly, after diagnosis, higher social status actually improves prognosis<sup>17;18</sup>. There could be a number of reasons for this phenomenon. Patients of higher socio-economic status tend to be more adherent to treatment, including neo-adjuvant and adjuvant therapies. Breast cancer discovered via screening mammography is likely to be at an earlier stage than those detected through imaging for symptomatic cancers, and will therefore carry a better prognosis. Patients in lower socio-economic groups are less likely to attend screening appointments, and may also delay seeking help even for clinically overt disease<sup>19</sup>.

Higher socio-economic status is often accompanied by an increased drive to learn about the illness and “red-flag” signs to watch for during recovery. These patients are also more willing to question doctors and ensure that all treatment avenues are explored.

Although not necessarily linked to socio-economic status, a slower decrease in breast cancer incidence has been reported in the rural USA as compared to urban areas<sup>20</sup>. This may represent saturation of mammography services in remote rural areas causing a backlog of undiagnosed cases which are still coming to light.

#### *1.1.2.2 Hormone Replacement Therapy and the Oral Contraceptive Pill*

That oestrogen can be a causative factor in breast cancer was first demonstrated in 1896 when bilateral oophorectomy induced remission in premenopausal women<sup>21</sup>.

Initial studies suggested a correlation between HRT and breast cancer<sup>22-24</sup> that swiftly vanished after cessation of treatment. These studies were validated when the incidence of oestrogen receptor positive (ER+) tumours fell from 2001 onwards in response to lower HRT prescription rates across the globe<sup>25-29</sup>. In the USA an 11.8% fall in incidence of ER+ tumours in those aged 50-69 between 2001 and 2004 was related to a massive decrease in prescription rates of two oestrogen-rich HRT preparations. Incidence of ER- tumours was unchanged<sup>30</sup>. Evidence published by the American Women’s Health Initiative described a 24% increased risk of breast cancer in post-menopausal women receiving conjugated oestrogens and medroxyprogesterone acetate<sup>31</sup>. The carcinogenic potential of oestrogen-only preparations is demonstrably less than that of combined alternatives<sup>32</sup>, and reducing the use of oestradiol-based preparations had no effect on cancer rates in Norway and Germany<sup>33;34</sup>. The World Health Organisation now adopts the view that the evidence for a causal link between combination HRT and breast cancer in post-menopausal women is incontrovertible<sup>35</sup>.

The oral contraceptive pill (OCP) is implicated as a carcinogen due to its oestrogen/progesterone content. The mitotic activity that such preparations induce in breast tissue may outweigh the protective benefits bestowed by anovulation<sup>36</sup>. Older studies simply relate use of the oral contraceptive to breast cancer without subgroup analysis<sup>37;38</sup>, while others suggest a stronger link where OCP use has been prolonged<sup>39</sup> or commenced at an early age<sup>39;40</sup>.

#### *1.1.2.3 Reproductive Behaviour and Endogenous Oestrogen*

Ovarian synthesis and secretion of oestrogen and progesterone begins at menarche and continues until the menopause. During the luteal phase of the menstrual cycle, increased circulating oestrogen and progesterone drive mitotic activity in the breast; it is during these phases of increased secretion that tumourigenic events occur<sup>41;42</sup>. Early menarche and late menopause prolong exposure to sex hormones and increase the risk of such events taking place<sup>43</sup>.

Most women undergo menarche within an age window of just a few years so effects on population incidence of breast cancer are limited. It is estimated that every 2 year delay in the onset of menarche confers a reduction in the relative risk of contracting breast cancer of around 10%<sup>44</sup>. In contrast, every additional year before initiation of the menopause is associated with a 2.8% increase in the risk of breast cancer<sup>22</sup>. The population-wide effects are thus dramatic, with a woman experiencing the menopause before the age of 40 having only a 50% chance of suffering breast cancer relative to one over 50<sup>22</sup>. These effects are no different between natural menopause and that surgically induced through bilateral oophorectomy<sup>22</sup>.

The first full-term pregnancy stimulates differentiation in the breast to a phenotype suitable for lactation<sup>45</sup>. This phenotype is more stable than “naive” mammary tissue, and much less susceptible to the carcinogenic effects of sex hormones. A full-term pregnancy at a young age



thus reduces breast cancer risk, whereas nulliparity increases it<sup>46,47</sup>. Multiple pregnancies and more time spent breast-feeding also reduce breast cancer risk<sup>48</sup>.

#### *1.1.2.4 Obesity*

Obesity is linked to a number of cancers, including colon and breast. The metabolic syndrome and insulin-resistance are also implicated; indeed the oral anti-diabetic preparation metformin has been shown to decrease overall cancer risk<sup>49</sup>. The action of metformin is mediated by AMP-kinase, which inhibits cellular proliferation by limiting the supply of lipid available for synthesis of new cell membranes<sup>50</sup>, and also directly blocks the cell cycle through induction of p53.

Obesity is also associated with increased plasma levels of leptin; an adipokine which acts as an appetite-suppressant, among other functions. It is thought that obese individuals fail to respond correctly to leptin, hence the increased circulating levels. High levels of leptin are significantly associated with breast cancer<sup>51</sup>. Leptin induces aromatase expression in MCF-7 breast cancer cells in culture<sup>52</sup>. Aromatase is a cytochrome p450 enzyme found in breast parenchyma that can catalyse oestrogen synthesis. Breast cancer in post-menopausal women may be due to local oestrogen synthesis, which would explain the increasing incidence of breast cancer with age, despite the lack of functioning ovaries<sup>53</sup>. Aromatase inhibitors are now widely-used in treatment of breast cancer worldwide.

#### *1.1.2.5 Alcohol*

Alcohol is a recognized risk factor for development of breast cancer<sup>54-56</sup>. Most experts agree that the underlying factor is oestrogen. The mechanism for this alcohol-oestrogen axis may be through alcohol-induced up-regulation of sex steroid synthesis<sup>57;58</sup> or inhibition of oestradiol clearance<sup>59</sup>. Additionally, ethanol up-regulates oestrogen receptor (ER)

expression<sup>60;61</sup>, proliferation of ER-positive cells (but not ER-negative)<sup>61</sup>, and activity of the ER- $\alpha$  receptor subgroup<sup>60</sup>. Other hypotheses implicate the ethanol metabolite acetaldehyde as a potential carcinogen<sup>62</sup>, as well as alcohol-induced reactive oxygen species and lipid peroxidation<sup>63</sup>. The higher incidence of breast cancer secondary to alcohol intake is limited exclusively to ER+ cancers<sup>64</sup>.

### **1.1.3 Genetic Risk Factors**

Having one first degree relative who has suffered breast cancer approximately doubles the relative risk for a given individual<sup>65</sup>. Familial relative risk increases when more relatives are affected<sup>65;66</sup>; and the sister of an affected monozygotic twin is more likely to contract breast cancer than that of a dizygotic twin<sup>67</sup>.

Around a third of ER-negative breast cancer is thought to be due to mutations in the BRCA1 (breast cancer 1) or BRCA2 genes, whereas roughly 10% of ER-positive breast cancer is caused by one of 12 recently discovered single nucleotide polymorphisms. Triple negative breast cancers (lacking oestrogen receptors, progesterone receptors or human epidermal growth factor receptor 2) are the only ones which do not demonstrate a familial preponderance<sup>68</sup>.

BRCA1 was linked to breast cancer susceptibility in 1994<sup>69</sup>; BRCA2 a year later<sup>70</sup>. Both are DNA repair genes which are also associated with increased incidence of ovarian cancer<sup>71</sup>. Family studies reveal that the likelihood of BRCA1/2 mutations increases both with numbers of breast or ovarian cancers in the family, and with lower ages at diagnosis<sup>72;73</sup>. They also suggest a penetrance of 50-85% for breast cancer by the age of 70 secondary to these mutations<sup>74;75</sup>. Both genes exhibit a range of potentially carcinogenic mutations, although the existence of populations displaying specific well-characterised mutations has allowed meaningful epidemiological research. 1% of Ashkenazi jews carry the 185delAG mutation in

BRCA1<sup>76</sup>; this is thought to be responsible for 20% of early onset breast cancer occurring within that population. 1.2% feature the 6174delT mutation in BRCA2 which accounts for a further 8% of cases<sup>77;78</sup>. In Iceland, the 999del5 mutation in BRCA2 is found in 24% of breast cancers diagnosed before the age of 40<sup>79</sup>. Penetrance estimates based on data from these populations are lower than from family studies; 56% of affected Ashkenazi jews would contract breast cancer by the age of 70<sup>80</sup>, as would 38% of affected Icelandic women<sup>81</sup>.

The ATM gene causes ataxia-telangiectasia (an autosomal-recessive condition)<sup>82</sup> and is associated with a 100-fold increase in cancers of all types. These cancers are predominantly lymphoid in childhood and epithelial in adults<sup>83</sup>. There have been over 300 mutations identified in the gene, with an overall prevalence of 0.5-1% in the Western world<sup>84</sup>. Heterozygotic ATM mutations cause a 2-fold increase in the risk of breast cancer, with a penetrance of 15%<sup>82</sup>.

CHEK2 is a cell cycle checkpoint kinase involved in mediating DNA damage-response pathways<sup>85</sup>. The 1100delC mutation doubles the risk of breast cancer<sup>86</sup>. It has also been reported to increase the risk of colorectal cancer<sup>87</sup>.

TP53 is a tumour suppressor gene encoding a transcription factor involved in cell cycling, apoptosis, DNA repair and genomic stability<sup>88</sup>. Mutations in TP53 cause the Li-Fraumeni syndrome; a genetic predisposition to breast cancer, as well as sarcomata and brain tumours<sup>89</sup>. Most mutations in TP53 are point mutations which prevent sequence-specific protein-DNA binding and inhibit p53 activation<sup>89;90</sup>.

CDH1 encodes E-cadherin, a glycoprotein involved in cell adhesion<sup>91</sup>. Mutations are implicated in familial diffuse gastric cancer; an autosomal dominant syndrome which confers around a 50% risk of lobular breast cancer<sup>92</sup>.

PTEN is a tumour suppressor gene encoding for phosphatidylinositol phosphate phosphatase. The protein product is involved in cellular regulation, although its exact role is unclear<sup>93</sup>. PTEN mutations may cause Cowden disease; an autosomal dominant syndrome pre-disposing patients to breast, thyroid and endometrial carcinomas and hamartomas<sup>94</sup>.

#### **1.1.4 Staging of Breast Cancer**

A staging protocol for breast cancer is given below<sup>95</sup>. Breast cancer is also staged using the TNM classification which takes into account extent of local tumour invasion, number and location of involved lymph nodes and the presence or absence of distant metastases.

<b>STAGE 0</b>	Ductal/lobular carcinoma in situ (DCIS/LCIS) No micro-invasion 5 year survival 92%
<b>STAGE I</b>	Invasive/ductal carcinoma of <2cm OR DCIS/LCIS with micro-invasion No lymph node involvement/metastases 5 year survival 87%
<b>STAGE II</b>	Invasive cancer of up to 5cm with up to 3 involved axillary lymph nodes OR Cancer of greater than 5cm diameter with no nodes involved 5 year survival 75%
<b>STAGE III</b>	Cancers up to 5cm with 4 or more involved axillary nodes OR Greater than 5 cm with any nodal involvement OR Any cancer with involvement of the ipsi-lateral internal mammary nodes OR Any skin involvement/chest wall fixation OR Any inflammatory cancer 5 year survival 46%
<b>STAGE IV</b>	Any cancer with distant metastasis 5 year survival 13%

#### **1.1.5 Histological Classification and Grading of Breast Cancer**

75% of neoplastic breast lesions are either DCIS (13%), invasive ductal carcinoma (55%), or invasive lobular carcinoma (5%)<sup>96</sup>. Medullary carcinomas also form in mammary tissue.

DCIS is a non-invasive condition; the term describes an accumulation of neoplastic cells within a lactiferous duct with no evidence of spread beyond it. Autopsy studies<sup>97</sup>, and studies on DCIS missed at biopsy<sup>98;99</sup>, suggest that lifetime risk of progression to invasive ductal carcinoma is substantially less than 50%.

Ductal carcinoma is further subdivided into cribriform, mucinous, tubular and papillary phenotypes, although the majority are unspecified. These four more differentiated forms carry a better prognosis. The breast may also be the site of other skin, connective tissue, vascular and neuroendocrine tumours.

Breast cancers are categorized according to histological findings – lobular carcinoma exhibits an arrangement of cells in large indistinct lobules. Ductal carcinomas retain a modicum of ductal architecture, albeit with massive disorganisation.

Breast cancers are assigned a histological grade, which is combined with stage at diagnosis to inform estimates of prognosis. The Bloom-Richardson scoring system rates cells according to 3 criteria: tubular structure formation; nuclear size, shape and staining intensity; and mitotic rate<sup>100</sup>. Cells can score up to 3 in each of these areas. Tumours are then described as low (scores of 5 or less), intermediate (6-7) or high (8-9) grade.

Histopathologists report evidence of vascular invasion when recording the details of specimens; this increases the risk of spread and suggests an increased likelihood of recurrence in the future, even in the absence of clinically detectable metastases at the time of staging.

Histopathologically also provides information on receptor status of breast cancers. All breast cancers are now described as positive or negative for oestrogen, progesterone and human epithelial growth factor receptors (ER, PR and HER2). Receptor positivity or negativity guides the selection of the adjuvant and neo-adjuvant biological, chemo- and hormonal therapy regimes used as adjuncts (or alternatives) to surgical resection of the primary lesion.

## 1.2 Iron

### 1.2.1 Normal Iron Homeostasis

Iron is an element crucial to many of the basic functions of the human body. Its ubiquity is due to the fact that it is a divalent metal that can easily switch between its ferric ( $\text{Fe}^{3+}$ ) and ferrous ( $\text{Fe}^{2+}$ ) states. This capability means it is easily able to donate and accept electrons in redox reactions<sup>101</sup>, including those responsible for oxygen transport, DNA biosynthesis, xenobiotic metabolism and oxidative phosphorylation<sup>102</sup>.

Paradoxically, it is the same chemical feature which is also responsible for the deleterious effects of excess body iron; as the majority of cytoplasmic iron is in the ferrous form, it readily donates electrons to undergo oxidation and generate free radicals. When this occurs in conjunction with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) or lipid peroxides as recipients, the resulting Fenton reaction will produce  $\text{Fe}^{3+}$ ,  $\text{OH}^-$  and the highly reactive hydroxyl ( $\cdot\text{OH}$ ) radical<sup>101</sup>. The reaction can compromise lipid membranes and lead to oxidative damage to DNA and other macromolecules<sup>103</sup> in an environment of so-called oxidative stress<sup>104;105</sup>.

The adult human body contains a total of 3-4g iron, of which around 70% is bound into the haem moieties of haemoglobin molecules in erythrocytes and their precursors<sup>106;107</sup>. To keep pace with erythrocyte destruction, 20-25mg<sup>103;107</sup> of iron is required per day to manufacture new haemoglobin in the bone marrow. Although the Western diet contains approximately 10mg iron per day, only 1-2mg of this is absorbed<sup>108</sup>; the remainder of the iron needed for haem synthesis is appropriated from the recycling of defunct red cells<sup>107</sup>.

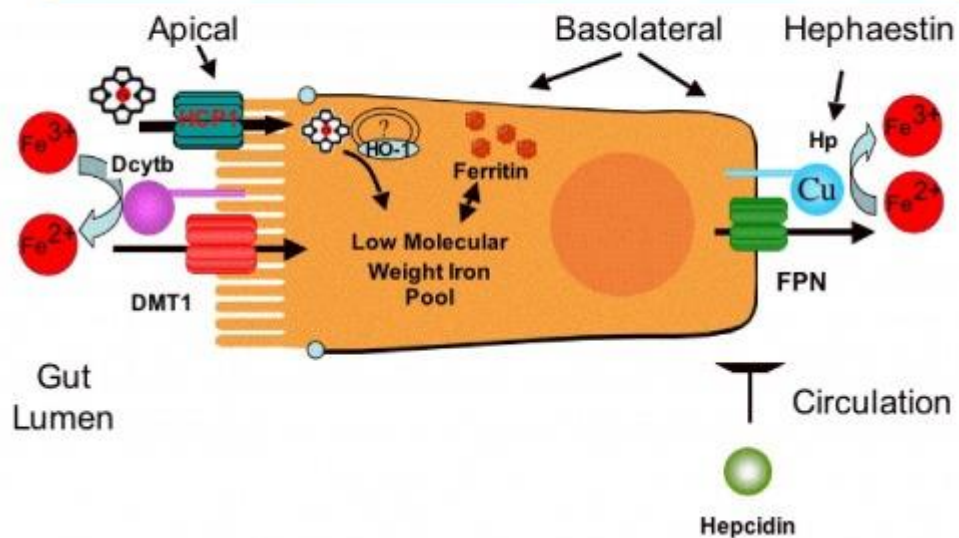
Given the potentially fatal consequences of both paucity and excess, optimal levels of circulating and stored iron are maintained by a finely balanced homeostatic mechanism.

Dietary iron is available as haem (mainly in red meat) and in its inorganic form. The pathways for absorption of each are illustrated in figures 1.1 and 1.4.

### **1.2.2 Non-haem Iron Metabolism**

Briefly, inorganic iron is imported from the gut lumen via the action of DMT1 (divalent metal transporter 1, a transmembrane shuttle), having first been reduced to  $\text{Fe}^{2+}$  by Dcytb (duodenal cytochrome b, a ferric reductase expressed on the luminal surface of duodenal enterocytes). Following import, iron is subject to one of three fates: 1) Stored bound to ferritin; 2) Utilised in one of the many intracellular processes requiring iron; 3) Exported from the enterocyte by ferroportin then oxidised by hephaestin for transport. Exported iron is then bound to transferrin and circulates around the body to be captured by cells expressing the transferrin receptor (TfR). The whole system is regulated by a number of different proteins, although the most important is likely to be the hormone hepcidin. The process is summarised below in figure 1.1. Individual proteins are discussed in detail thereafter.

## Iron Transport through the Enterocyte



**Figure 1.1 Import of Dietary Iron from the Duodenum**

Dietary ferrous iron is reduced by Dcytb to its ferric form for import via DMT1. If not utilised by the cell, the iron is then stored bound to ferritin, enters the labile iron pool or is cycled through the cell and exported by ferroportin. Ferroportin requires the action of hephaestin to re-oxidise the iron after export such that it can bind to transferrin for transport around the body. HCP1 is an importer of haem iron, discussed in due course (*original diagram C Vulpe; University of California*).



#### *1.2.2.1 Duodenal Cytochrome b (Dcytb)*

Inorganic (non-haem) iron is found mainly in grains and vegetables and is absorbed by active transport from the gut lumen. The protein responsible for this active transport is DMT1 (Divalent Metal Transporter 1), but it is only capable of transporting iron in its ferrous ( $\text{Fe}^{2+}$ ) form. Owing to the low luminal pH of the upper gastrointestinal tract, dietary inorganic iron is usually to be found in the ferric ( $\text{Fe}^{3+}$ ) state<sup>109</sup> and must therefore be reduced prior to absorption. The duodenum was found to have a ferric reductase activity in 1992<sup>110</sup>, but Dcytb was not formally identified as the protein responsible until 2001. It is a di-haem protein located in the upper villous parts of the duodenal brush border, regulated in response to systemic iron levels. Anaemia induces an up-regulation of expression to facilitate further absorption of iron<sup>109</sup>. Murine studies demonstrate increased levels of Dcytb in a haemochromatosis (iron overload) mouse model, along with DMT1<sup>111</sup>. However, further murine studies report normal iron uptake in mice lacking Cybrd1, the murine homolog of Dcytb<sup>112</sup>, raising the question of whether Dcytb is in fact essential for iron uptake from the gut or whether other import mechanisms exist.

In terms of structure and membrane topology the protein strongly resembles the trans-membrane electron shuttle cytochrome b561. Cytochrome b561 uses semidehydroascorbic acid as a substrate to accept electrons<sup>113</sup>; the binding site for this is preserved in Dcytb suggesting a similar mechanism for its reductase function.

#### *1.2.2.2 Divalent Metal Transporter 1 (DMT1)*

DMT1 (Nramp2, SCL11A2) was first described in 1997 by two separate groups<sup>114;115</sup>. Initially identified owing to a gene mutation in the mutant microcytic anaemia (mk) mouse<sup>114</sup>, it has since been shown to be a glycoprotein featuring 12 membrane-spanning domains and

displaying marked hydrophobicity. It functions as an ion transporter for a range of divalent substrates including cadmium, lead, zinc, manganese, copper and cobalt<sup>116</sup>.

The mutation seen in one of the trans-membrane domains in the mk mouse (Gly185Arg) has also been identified in the anaemic Belgrade rat<sup>117</sup>, further confirming the role of DMT1 as an iron importer.

DMT1 exists in 4 different isoforms owing to splice variants at either the 3' or 5' end. At the 5' end alternative splicing generates 2 initiation points for transcription at exons 1A and 1B, while at the 3' end it is responsible for the inclusion or omission of an iron-response element (IRE)<sup>118</sup>. The 1A isoform contains an extra 29 amino acids at the 5' end. The IRE in IRE+ DMT1 is contained within the 3' un-translated region (UTR). Some of the other iron transport proteins (ferroportin<sup>119;120</sup>, TfR<sup>105</sup> and ferritin<sup>105</sup>) also feature IREs. These are expressed in the 3' UTR in DMT1 and TfR1, when the aim of interaction with iron regulatory proteins is to up-regulate expression, and the 5' UTR in ferritin and ferroportin when interaction is designed to down-regulate expression. Iron regulatory proteins are discussed in detail in section 1.2.2.8.

All 4 DMT1 isoforms function with equal efficacy as iron transporters; the differences in structure allow for variation in location and regulation according to the requirements of different tissues<sup>121</sup>. DMT1A is predominantly found on the apical membranes of enterocytes throughout the intestine, primarily focussed in the duodenum. DMT1B is ubiquitously expressed, although it is especially prevalent in red cells and is largely cytosolic<sup>122;123</sup>. It is therefore likely that DMT1A functions mainly as a duodenal iron importer, whereas DMT1B facilitates extrusion of iron from intracellular endosomes following endocytosis of transferrin-bound iron (discussed later)<sup>115</sup>.

The IREs are used to regulate expression of DMT1 in response to systemic iron levels. Under normal circumstances intestinal expression of DMT1 is kept at a low level, but this is up-

regulated when dietary iron is deficient and intracellular concentrations are low<sup>122;124</sup>. This up-regulation is achieved through induction of the iron response proteins IRP1 and IRP2 which bind to the IREs and stabilise DMT1 mRNA, thus increasing DMT1 protein expression and iron uptake from the gut<sup>105</sup>. IRP1 and IRP2 are further discussed in section 1.2.2.8.

### *1.2.2.3 Ferritin*

Following absorption from the duodenal lumen, iron may be utilised in enterocytes, exported to the circulation or stored. Ferritin is the protein to which iron binds for storage and is ubiquitously expressed throughout all tissue types (in multiple intracellular locations) and in the serum<sup>125-128</sup>. This storage facility sequesters a potentially harmful intracellular “labile iron pool” of ferrous iron<sup>129</sup>. Ferritin consists of 24 subunits arranged in a globular shell-like structure<sup>130</sup>. These subunits are either heavy (H) or light (L); the H and L subunits are encoded by different genes and the ratio of H to L is tissue-dependent<sup>131;132</sup>. Ferritin H is profusely expressed in cardiac tissue, whereas the liver and spleen exhibit a preponderance of ferritin L<sup>125</sup>. The H subunit deploys a ferroxidase activity to oxidise the ferrous iron imported by DMT1 back into its ferric ( $\text{Fe}^{3+}$ ) form for binding<sup>129</sup>. The L subunit stabilises the shell structure and facilitates uptake of ferric iron into it<sup>133</sup>. Each ferritin protein is capable of sequestering 4500 iron atoms<sup>125</sup>.

The mechanism of action of ferritin lends it anti-oxidant properties. Induction of ferritin H by NF- $\kappa$ B via TNF- $\alpha$  suppresses the production of reactive oxygen species (ROS) from Fenton reaction chemistry, thus inhibiting apoptosis<sup>134</sup>. Murine studies have demonstrated reduced oxidative stress in mice in which ferritin H has been genetically upregulated<sup>135</sup>. Tissue culture experiments have shown that HeLa and murine erythrolukaemia cells that over-express ferritin accumulate less labile iron and ROS<sup>136;137</sup>; conversely use of siRNA to disrupt ferritin H in the same murine cells induced oxidative stress<sup>138</sup>.

Ferritin is also regulated by IRP1 and IRP2. The IRE of ferritin is found in the 5' UTR of its mRNA<sup>139</sup>. Excess iron induces IRP1 to assemble a cubane iron-sulphur cluster (ISC), the binding site for which overlaps that for the H ferritin IRE. Thus, when iron levels increase and more storage capacity is needed to avoid high levels of labile iron, IRP1 is prevented from binding to the ferritin IRE. Because IRP1 is inhibitory to translation of ferritin mRNA, reduced IRP-IRE interaction is permissive for up-regulation of ferritin protein to buffer the increasing numbers of free Fe<sup>2+</sup> ions<sup>105;140</sup>. Similarly, when cells are iron replete, IRP2 undergoes proteasomal degradation, again allowing ferritin to be expressed at higher concentrations<sup>105</sup>. In murine knockout studies, IRP1 knockout mice demonstrated very little dysregulation in iron homeostasis, whereas IRP2 knockouts displayed abnormal ferritin induction and consequent abnormalities in iron handling. This suggests that IRP2 is in fact the primary factor in control of ferritin regulation<sup>141;142</sup>.

Ferritin is also regulated in response to changing concentrations of haem. Friend Leukaemia Cells induced to differentiate by exogenous haem were observed to up-regulate ferritin expression via activation of NF-Y and p300<sup>143;144</sup>. NF-Y binds to a site approximately 100bp upstream from the initiation site for ferritin H transcription, and the haem-responsive element in the ferritin promoter region has been shown to overlap with the anti-oxidant response elements of both ferritin L and H which allow ferritin to act as an anti-oxidant in times of oxidative stress<sup>145;146</sup>.

#### *1.2.2.4 Ferroportin*

Ferroportin (IREG-1, MTP-1) was discovered by two independent groups; one identified a protein termed ferroportin 1 in mutant zebrafish, where gene mutations conferred a state of hypochromic anaemia<sup>119</sup>, the second identified iron regulatory gene-1 in hypotransferrinaemic (sla) mice and later cloned the protein they named metal transport protein-1<sup>120</sup>. Ferroportin is

now the universally accepted name for this transporter, which is the only exporter of inorganic iron found to date.

Ferroportin is a 571 amino acid protein with multiple transmembrane domains. It is densely expressed in human liver, kidney, spleen, heart, placenta and enterocytes<sup>120;147</sup>. Expression is far more abundant on the basement membranes of polarized duodenal enterocytes in particular, consistent with a role in the export of iron from these cells<sup>120</sup>.

The ability of ferroportin to export iron out of cells has been demonstrated by in vitro tissue culture experiments. Reduced cytosolic iron levels were reported following artificially induced over-expression of ferroportin<sup>124</sup>, while viral transfection to over-express ferroportin in a murine macrophage cell line led to a 70% increase in efflux of <sup>59</sup>Fe after phagocytosis of <sup>59</sup>Fe-labelled erythrocytes<sup>148</sup>.

Regulation of ferroportin is achieved via two distinct mechanisms. The first is reliant on the fact that ferroportin mRNA also features an IRE, in the 5' UTR. At low iron concentrations the IRP-IRE interaction would act to inhibit ferroportin expression in the same manner seen with ferritin to increase intracellular availability<sup>120</sup>. However, the primary system for ferroportin regulation is through the action of the hormone hepcidin. Now regarded as a master-regulator of iron metabolism, hepcidin binds to and phosphorylates ferroportin, which is then internalized, de-phosphorylated, ubiquitinated and directed for lysosomal degradation<sup>149-151</sup>. Hepcidin is synthesized in the liver<sup>152</sup>; its expression is up-regulated in response to increasing iron levels and inflammation, and decreased by hypoxia and anaemia<sup>153;154</sup>. Hepcidin is discussed further in section 1.2.2.6.

As might be expected, mutations in the gene which codes for ferroportin can lead to iron overload due to autosomal dominant forms of haemochromatosis unrelated to mutations in the HFE gene. The A77D missense mutation generates a disease phenotype characterized by early

iron overload in reticuloendocytic macrophages. This form of haemochromatosis comes about because of localisation of ferroportin to the cytosol rather than the cell membrane, depriving macrophages of their capability to extrude iron<sup>155;156</sup>. The N144H missense mutation results in the classic phenotype of hereditary haemochromatosis in which the functionality of enterocytic ferroportin is augmented by a lack of response to inhibitory hepcidin, thus increasing efflux of iron from the intestines<sup>157;158</sup>. In this variation of the disease there is a high concentration of serum transferrin and vastly increased liver deposits of iron<sup>158</sup>.

#### *1.2.2.5 Hephaestin*

Ferroportin exports iron from cells in the ferrous ( $\text{Fe}^{2+}$ ) form. However, iron is transported around the body bound to transferrin in its ferric state. In many cell types, including glioma cells, astrocytes and macrophages, the multi-copper oxidase caeruloplasmin (Cp) converts divalent iron into trivalent for transport<sup>159-161</sup>. The human condition acaeruloplasminaemia (lack of Cp) results in iron accumulation in the retina, basal ganglia, spleen, liver and pancreas<sup>162</sup>. Murine studies on a Cp-knockout revealed similar hepatic and splenic iron overload, as well as high iron levels in cells of the reticuloendothelial system<sup>160</sup>.

Caeruloplasmin is not found in the intestine; its role there is filled by a transmembrane-bound homologue named hephaestin. This first came to light following studies on the sex-linked anaemia (Sla) mouse, members of which population exhibit a microcytic anaemia consequent upon impaired efflux of iron absorbed (in the normal manner) by the intestines<sup>163</sup>. The impairment in function is due to a 582 nucleotide deletion in the hephaestin gene which would code for a 194 amino acid sequence in its protein product<sup>163</sup>. Hephaestin and ferroportin expression are closely related, with marked co-localisation on the basement membranes of the duodenal enterocytes responsible for iron uptake<sup>164</sup>.

The mechanisms by which hephaestin expression is regulated are unclear. Hephaestin is known to be up-regulated by the homeobox transcription factor CDX2, whose main role is in regulating intestinal development and differentiation. High levels of intracellular iron induce CDX2, with the resulting expression of hephaestin leading to increased iron efflux and restoration of normal iron levels<sup>165</sup>. However, an alternative hypothesis proposes that hephaestin is regulated in response to systemic iron levels rather than at the cellular level; in this model, systemic shortages of iron trigger up-regulation of both ferroportin and hephaestin, with the consequent fall in intracellular iron levels causing compensatory up-regulation of DMT1 to increase overall body stores<sup>166</sup>.

#### *1.2.2.6 Hepcidin*

Hepcidin was identified in 2000-2001 when a number of groups isolated it independently of each other in plasma<sup>167</sup>, urine<sup>152</sup> and liver<sup>153</sup>. Murine studies demonstrate iron overload in USF2 (upstream stimulatory factor 2) knockout mice, which also lack the hepcidin gene<sup>168</sup>, and severe anaemia in mouse models in which hepcidin is over-expressed<sup>169</sup>. Administration of synthetic hepcidin also induces hypoferraemia in mouse models<sup>170</sup>. Hepcidin is a 25 amino-acid peptide synthesized in the liver as an 84 amino-acid precursor<sup>152</sup>. It has hydrophilic and hydrophobic elements, enabling it to function as an anti-microbial (in fact its original name was liver-expressed anti-microbial peptide or LEAP-1)<sup>171</sup>. It is known to be expressed in response to increasing systemic iron levels and inflammation<sup>153</sup>, while its expression is down-regulated in response to hypoxia and anaemia<sup>154</sup>.

As described earlier, hepcidin has been shown to bind to ferroportin to trigger its internalization and lysosomal degradation<sup>150</sup>. It is accepted that the active part of the hepcidin peptide in this context is the N-terminus; deletion of the 5 amino-acids positioned here

completely abrogates the decrease in iron levels usually seen when hepcidin is over-expressed<sup>172</sup>.

Hepcidin is now routinely described as the master-regulator of systemic iron levels<sup>173</sup>, with iron overload inducing hepatic hepcidin mRNA<sup>153</sup>. Hepcidin mRNA does not appear to contain the IRE seen in many of the other iron-regulatory proteins<sup>174</sup>, so an alternative method of regulation must be operating. The discovery of CCAAT/enhancer-binding protein (C/EBP) sequences in the hepcidin promoter region, as well as signal transducer and activator of transcription (STAT) motifs in its DNA, suggest that expression may be modulated at the transcriptional level<sup>175</sup>. C/EBP $\alpha$  in particular is implicated; iron loading doubles hepatic expression of C/EBP $\alpha$  in murine liver, and C/EBP $\alpha$ -deficient mice possess lower levels of hepcidin mRNA<sup>175</sup>. How iron can stimulate C/EBP $\alpha$  to initiate the hepcidin-ferroportin regulatory axis has yet to be elucidated.

The gene associated with hereditary haemochromatosis (HFE) is also implicated in control of hepcidin expression. HFE knockout mice exhibit no up-regulation of hepcidin mRNA in response to increased iron loading, leading to hepatic deposition of iron and increased transferrin saturation<sup>176</sup>. Reconstituting hepcidin in these knockouts restored a normal response to iron loading and normalised the haemochromatosis phenotype<sup>177</sup>. Human studies comparing haemochromatosis patients to normal individuals revealed a similar picture of failure of hepcidin up-regulation in response to increasing serum iron levels and over-expression of ferroportin<sup>178</sup>.

Hemojuvelin, otherwise known as HFE2, has also been shown to up-regulate hepcidin expression<sup>179</sup>; mutations therein are thought to be responsible for juvenile haemochromatosis<sup>180</sup>. HFE2 acts through a signalling pathway involving bone morphogenetic proteins (BMPs). BMPs are members of the TGF $\beta$  superfamily which act through Smad



proteins and MAP kinases, and have been demonstrated to stimulate hepcidin production<sup>179</sup>. HFE2 mutations suppress BMP signalling, and Smad4 knockout mice exhibit lower levels of hepcidin in response to iron loading<sup>179</sup>.

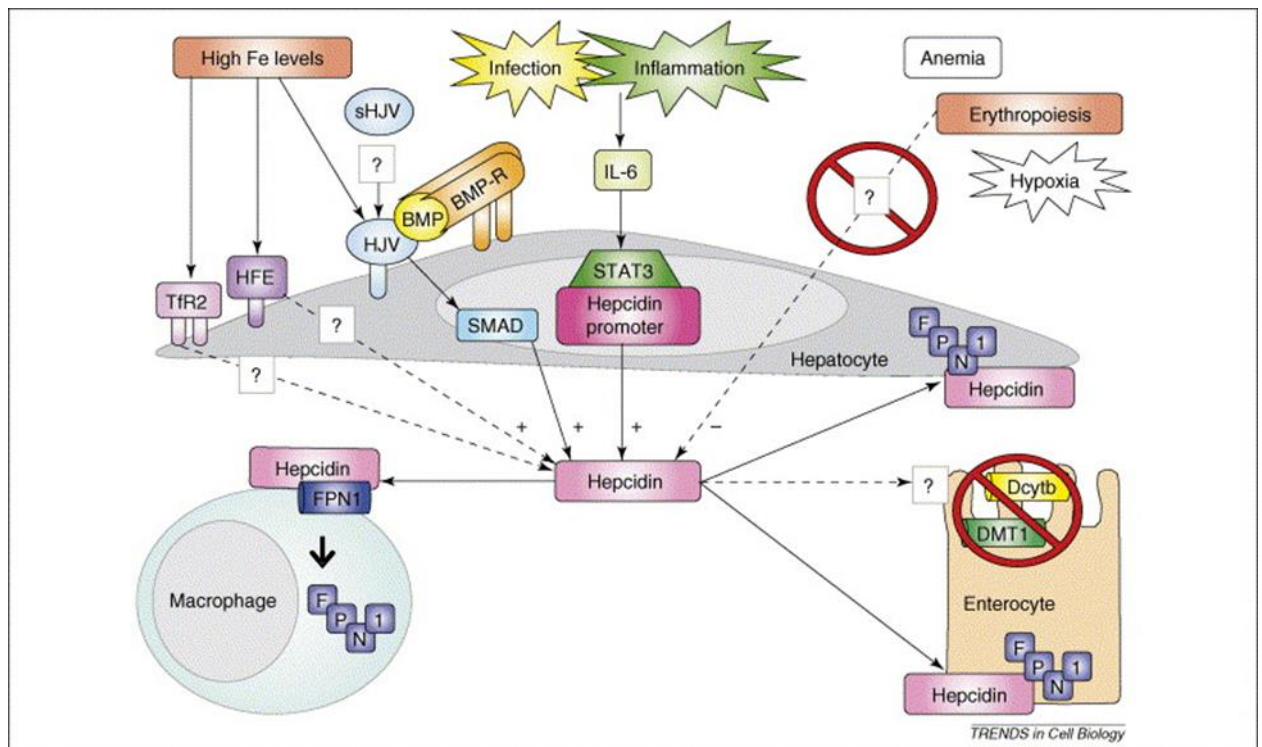
Transferrin receptor 2 (TfR2) mutations confer a similar phenotype to those seen in conjunction with HFE and hemojuvelin abnormalities<sup>181;182</sup>. This suggests a potential role for TfR2 in hepcidin regulation in addition to its role as an importer of iron from the circulation into hepatocytes<sup>183;184</sup>.

Murine studies have also implicated hepcidin in the development of the anaemia of chronic inflammation/disease<sup>185;186</sup>. The induction of hepcidin expression by inflammation is mediated via the activity of IL-6<sup>185</sup>. Antibodies against IL-6 counteract the up-regulation of hepcidin mRNA expression in murine hepatocytes seen during inflammation<sup>153</sup> and IL-6 knockout mice are incapable of generating this response<sup>185</sup>. IL-6 effects hepcidin up-regulation via activation of STAT3<sup>187</sup>, which subsequently interacts with one of the STAT binding sites in the hepcidin gene promoter region<sup>188;189</sup>.

Leptin is also implicated in the induction of hepcidin expression. Human hepatoma (HuH7) cells incubated with leptin exhibit marked over-expression of hepcidin mRNA<sup>190</sup>. Obese individuals whose leptin levels are high display deranged iron homeostasis.

Finally, hepcidin is also regulated in response to hypoxia, with lower oxygen tension resulting in down-regulation of hepcidin expression<sup>154;191</sup>. This is mediated by hypoxia-inducible factor (HIF-1); hypoxia causes accumulation of cytoplasmic HIF-1 $\alpha$  which then relocates to the nucleus and binds to HIF-1 $\beta$ . The resulting complex binds to a hypoxia responsive element in hepcidin DNA to down-regulate transcription<sup>191</sup>. The resulting increase in iron efflux from cells permits increased erythropoiesis to generate increased capacity for oxygen carriage. The existence of this axis for hypoxia-mediated control of hepcidin expression has been proven by

various murine studies. Knockout mice lacking von Hippel-Lindau (a negative regulator of HIF-1) display increased ferroportin expression, while HIF-1 $\alpha$  knockouts exhibit augmented hepcidin expression in response to hypoferraemia<sup>191</sup>. The actions and regulation of hepcidin are summarised diagrammatically in figure 1.2.

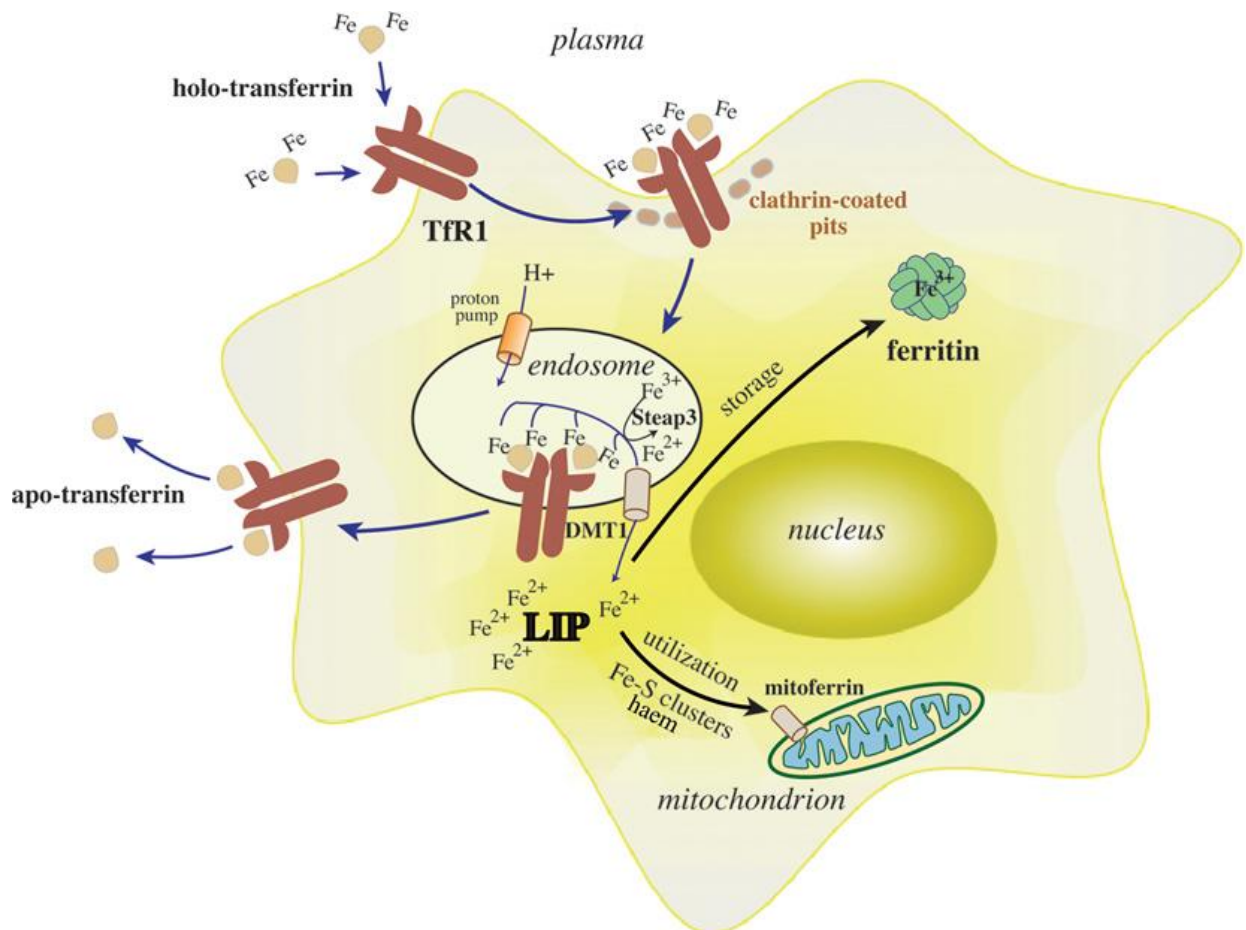


**Figure 1.2 Regulation and actions of hepcidin**

Increased iron, infection and inflammation induce hepcidin expression, which then exerts an inhibitory effect on iron export from cells by suppression of ferroportin activity (*original diagram LL Dunn; Trends Cell Biol*).

### 1.2.2.7 Transferrin Receptor

The transferrin receptor (TfR) binds transferrin-bound iron for absorption into destination cells via receptor-mediated endocytosis. The process is summarised in figure 1.3 and described in detail below.



**Figure 1.3 TfR-Mediated Endocytosis**

The iron-transferrin complex binds to TfR and undergoes receptor-mediated endocytosis. Acidification of the endosome liberates  $\text{Fe}^{3+}$  which is reduced by Steap3.  $\text{Fe}^{2+}$  is exported to the cytoplasm by DMT1 for utilization, storage or entry into the labile iron pool (*original diagram K Pantopoulos; Biochem J*).

Transferrin is the protein to which ferric iron binds for transport around the body in the circulation<sup>192</sup>. It is synthesized and secreted by the liver<sup>193</sup> as a hinged bi-lobar structure; the cleft between the two lobes can bind two iron atoms for delivery to the tissues<sup>194</sup>. Transferrin binds to the transferrin receptor (TfR) expressed on the cell membranes of destination tissue, where receptor-mediated endocytosis internalizes the complex<sup>192</sup>. In common with many such internalization mechanisms, the vesicle is coated in a proteinaceous coat of clathrin which helps to form the vesicle and package its contents<sup>195</sup>. A conformational change in transferrin is induced by the acid environment of the endosome to release iron<sup>115</sup> whereupon it is reduced to  $\text{Fe}^{2+}$  by the ferric reductase Steap3<sup>196</sup> and transported from endosome to cytoplasm by DMT1B<sup>115</sup>.

TfR exists in 2 forms; TfR1 and TfR2<sup>197</sup>. TfR1 is expressed throughout the body and has a high affinity for the transferrin-Fe complex. TfR2 is localised to the liver and intestinal iron-absorbing cells<sup>198</sup> and demonstrates lower affinity<sup>197;199</sup>. TfR is expressed as a homodimer, each half being composed of an extracellular stalk, a transmembrane segment and a cytoplasmic domain. Transferrin binds across the dimer<sup>200</sup>, triggering an internalization signal emanating from a tyrosine near the cytoplasmic N-terminus of TfR<sup>201</sup>.

TfR expression is regulated by iron levels and hypoxia. In common with many of the other iron transport proteins, TfR mRNA possesses IREs; in this case 5 of them, all located in the 3' UTR<sup>202</sup>. Thus, IRP binding stabilises the mRNA permitting increased translation in times of iron shortage to allow increased import from the serum<sup>139</sup>.

Hypoxia regulates TfR at the transcriptional level. TfR promoter DNA contains an HRE which (in contrast to the inhibitory HRE found in hepcidin mRNA) up-regulates transcription when hypoxia causes HIF-1 $\alpha$  and HIF-1 $\beta$  to complex and bind to it<sup>203;204</sup>. In vitro studies on K562 (hepatoma) cells and HeLa (human cervical carcinoma) cells demonstrated up-regulation of

TfR1 mRNA in response to hypoxia, abrogation of this response when the HIF-1 binding site in the HRE was mutated, and enhanced TfR1 promoter activity in the face of over-expression of the HIF-1 $\alpha$ / $\beta$  subunits<sup>203;205</sup>. Iron chelators have been found to increase TfR1 transcription in HepB3 human hepatoma cells, which response was again abrogated following mutation of the HRE or in the presence of dysfunctional HIF-1 $\alpha$ <sup>206</sup>.

#### *1.2.2.8 Iron Regulatory Elements and the Iron Response Proteins*

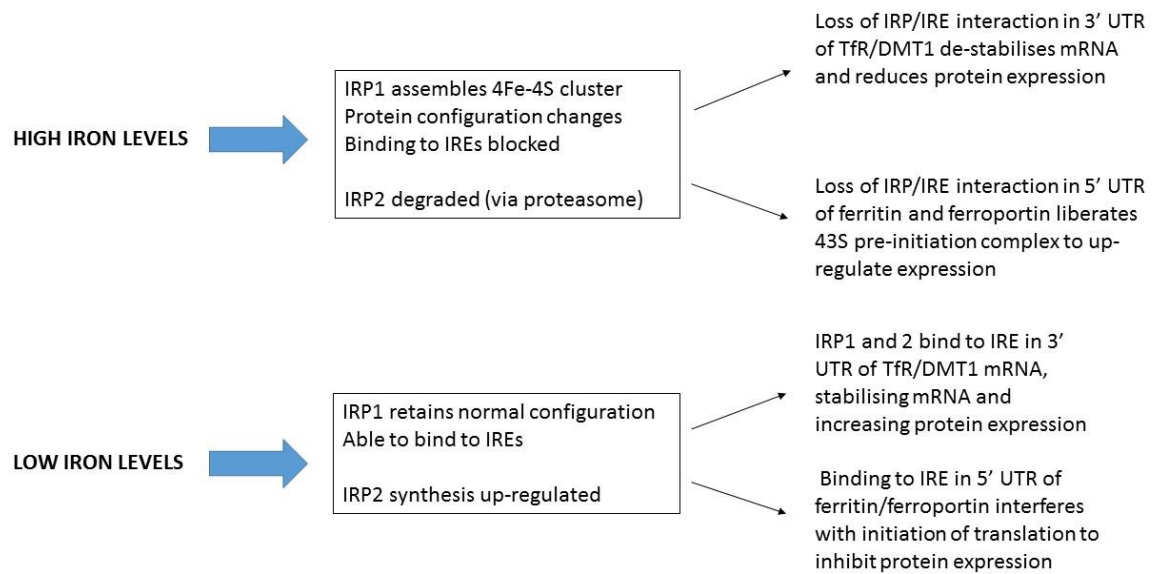
As has already been mentioned, several of the proteins involved in iron handling incorporate iron-responsive elements (IREs) in un-translated regions of their mRNA to allow post-transcriptional regulation of expression in response to intracellular iron levels<sup>105;118-120;202</sup>. These IREs can occur in either the 3' or 5' UTR of the mRNA and their location is of functional importance<sup>207</sup>. With an IRE at the 3' end, binding of an iron response protein (IRP) to it stabilises the mRNA to up-regulate translation, thereby increasing expression of the protein end-product. Conversely, IRP-IRE interaction at the 5' end interferes with assembly of the 43S pre-initiation complex to inhibit protein expression<sup>208</sup>. As can be inferred, proteins with 3' IREs include TfR and DMT1, whereas ferroportin and ferritin (both L and H) feature IREs at the 5' end<sup>103;105</sup>. Hence, induction of the IRE-IRP system will increase influx of iron from the gut or circulation, whilst liberating intracellular stores and preventing efflux.

There are 2 IRPs, both members of the iron-sulfur cluster isomerase family<sup>209</sup>. Both are thought to be formed of 4 domains; 3 closely linked with the 4<sup>th</sup> attached by a flexible hinge mechanism<sup>105</sup>. The major difference is that IRP-2 contains a 73 amino-acid insertion in domain 1, encoded by a unique exon, which is rich in cysteine and proline.

Both IRP1 and IRP2 are ubiquitously expressed<sup>105</sup> and do not appear to differ in their relative capacities to influence regulation of iron transport proteins<sup>210-212</sup>. However, cell culture studies have demonstrated that proportional responsibility for regulation may differ between tissue

types. In murine J774 cells<sup>213</sup> and RAW macrophages<sup>214;215</sup>, only IRP2 modulation was seen to affect TfR and ferritin expression, with no part played by IRP1. The situation was reversed in human RD4 (rhabdomyosarcoma)<sup>216</sup> and H1299 (lung cancer)<sup>217</sup> cells where expression of the mutant IRP1<sub>C437S</sub> led to dysregulation of the same two proteins.

Regulation of IRP1 and IRP2 is achieved through 2 distinct mechanisms. When iron is plentiful, IRP1 assembles a 4Fe-4S cluster<sup>218</sup> which prevents normal binding to IREs and confers upon IRP1 the activity of a cytosolic aconitase, which may play a role in redox control<sup>219</sup>. Iron depletion induces disassembly of the cluster<sup>220</sup>, which probably reorganises the cleft between domains 1-3 and the hinged domain 4, providing more space therein and restoring IRE-binding potential<sup>221</sup>. The iron-sulphur cluster is also induced to disassemble in times of oxidative stress in response to H<sub>2</sub>O<sub>2</sub> and NO<sup>222;223</sup>, which may implicate IRP1 in the inflammatory response and the anaemia of chronic disease (by increasing cellular import of iron and down-regulating export, leading to decreased levels of circulating iron). The regulation of IRP2 is markedly different – it is synthesized de novo when iron is in short supply<sup>224</sup> and undergoes proteasomal degradation when iron levels increase<sup>225</sup>. The control mechanism was originally thought to rely on the 73 amino acid insertion described earlier. It was proposed that this domain functions as an iron sensor, and that the binding of iron to it induces oxidation of 3 cysteine residues (C168, C174 and C178), rendering IRP2 recognisable to the proteasome and marking it for degradation<sup>226</sup>. Recent work has demonstrated that mutations in these 3 residues<sup>227</sup>, or even deletion of the entire insert<sup>227;228</sup>, does not protect IRP2 from degradation, although an alternative method for its down-regulation in iron-replete cells has not yet been delineated.



**Figure 1.4 The Role of IRP1 and IRP2 in Regulation of Intracellular Iron Levels**

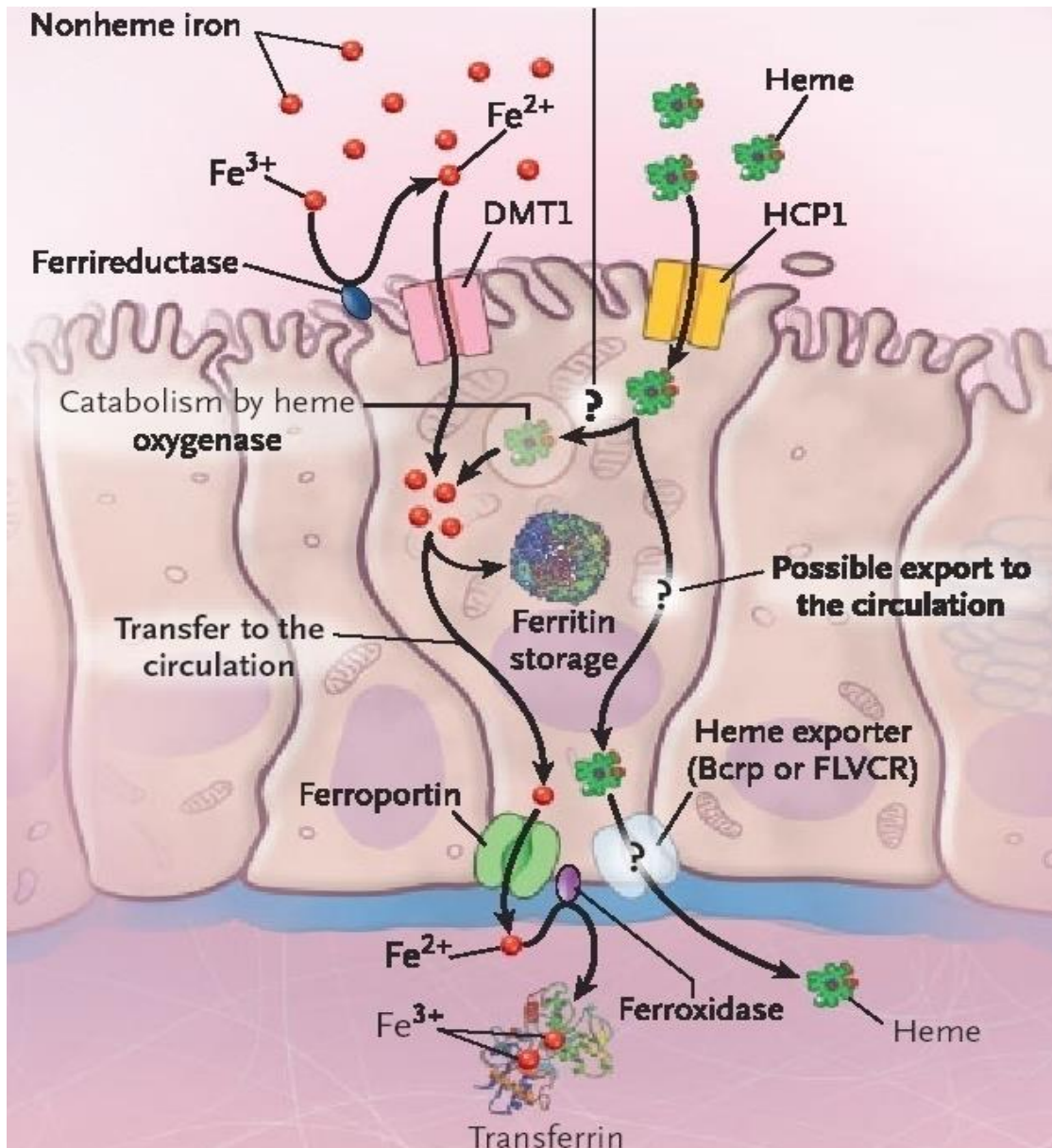
High iron concentrations reduce IRP/IRE interaction to encourage intracellular sequestration by ferritin and efflux by ferroportin. At low iron concentrations increased IRP/IRE interaction blocks iron efflux, reduces binding to ferritin and encourages import from the circulation via TfR and DMT1.

### 1.2.3 Haem Metabolism

Haem (iron-protoporphyrin IX) is less common in the diet than the inorganic iron previously discussed, being confined to red meat, but it is more efficiently absorbed. It has a key role to play in the function of haemoprotein enzymes such as catalase, peroxidase, cytochromes c and p450 (electron transfer and drug metabolism), nitric oxide synthase (signal transduction) and NADPH oxidase. Disordered haem metabolism is implicated in the pathogenesis of haematological disorders such as porphyria and sickle-cell disease. It is also a component of haemoglobin, myoglobin, cytoglobin and neuroglobin and has been shown to be involved in regulation of transcription, translation and cellular differentiation<sup>229;230</sup>. Because free haem is potentially cytotoxic (much like ferrous iron, it can generate harmful reactive oxygen species), it is tightly controlled to maintain a low intracellular concentration. This is achieved (in non-erythrocytes at least) through the negative feedback effect of haem on the synthetic enzyme  $\delta$ -aminolaevulinic acid synthase (ALAS), and its positive effects on expression of catabolic haem oxygenase-1 (HO-1)<sup>231-233</sup>.

Although a multitude of proteins involved in the handling of inorganic iron, from the GI tract to the peripheral tissues, have been identified and exhaustively researched, relatively little was known until recently about mechanisms of haem transport into and out of cells. HCP-1 (haem carrier protein 1) has now been identified as a haem importer protein, and LRP1 (lipoprotein receptor-related protein 1) is thought to have a similar role. Haem within cells can either be broken down by haem-oxygenase-1 (HO-1) into free iron and biliverdin (then reduced to bilirubin), or trafficked through cells and exported by FLVCR (feline leukemia virus subgroup C receptor) or BCRP (breast cancer resistance protein, also known as ABCG2). Haem transport is summarised below in figure 1.4. The individual proteins involved are discussed in greater detail thereafter.





**Figure 1.5 Iron and haem transport in the enterocyte**

Inorganic iron transport is shown on the left side of the above illustration, haem transport on the right. HCP-1 has been shown to import haem; LRP1 is likely to share this role. HO-1 is capable of catabolising haem to release inorganic iron which then enters the normal intracellular iron-handling machinery as shown. FLVCR and BCRP (ABCG2) are both implicated in haem export.

#### *1.2.3.1 Haem Carrier Protein 1 (HCP-1)*

It is recognised that haem, as a lipophilic molecule, is capable of diffusing across cell membranes without the need for active transport<sup>234</sup>. Initial evidence for the existence of an unspecified transporter able to import haem came from studies on intestinal Caco-2, and non-intestinal K562 (human leukaemia) and HepG2 (human hepatocellular carcinoma) cells, reporting energy-dependent haem uptake<sup>235;236</sup>. This work followed the demonstration of an intestinal brush border haem receptor in both porcine<sup>237</sup> and human<sup>235</sup> gut, although at the time it could not be characterized. While some researchers postulated that haem was degraded in the gut lumen prior to absorption<sup>238</sup>, electron microscopy has clearly illustrated the presence of haem porphyrin rings in micro-endocytic vesicles in the bases of gut microvilli and in tubovesicular structures in the adjacent cytoplasm of the epithelial cells<sup>239</sup>.

HCP-1 was isolated from mouse duodenum via subtractive suppression hybridization<sup>240</sup>. It is primarily expressed in the duodenum, but can also be found in liver and kidney<sup>240</sup>, and is expressed by macrophages<sup>241</sup>. It is a 446 amino acid protein exhibiting marked hydrophobic tendencies, with 9 transmembrane domains<sup>240</sup>.

Varied evidence exists to demonstrate that HCP-1 functions as a haem transporter. *Xenopus* oocytes and cultured cells both exhibit 2-3-fold increase in haem uptake secondary to HCP-1 expression, and HCP-1 also facilitates uptake of the structurally homologous Zn-protoporphyrin. Conversely, uptake of radio-labelled haem by duodenal cells was inhibited in the presence of anti-HCP-1 antibodies<sup>230</sup>.

Regulation of HCP-1 occurs at both transcriptional and post-translational levels. HCP-1 mRNA is up-regulated by hypoxia and hypotransferrinaemia<sup>230</sup> through a regulatory mechanism that has yet to be clarified. Post-translational regulation is better understood. Before the discovery of HCP-1, rodent studies had already described an increase in haem

absorption in response to iron-deficiency<sup>242</sup>. The group responsible for identifying HCP-1 also determined that its location within the cell changes in response to iron load; iron-replete rats sequester HCP-1 in the cytoplasm, whereas it is translocated to the apical membrane when they are rendered iron-deficient<sup>240</sup>.

The activity of HO-1 may also be related to HCP-1 expression. Iron deficiency induces up-regulation of HO-1<sup>243</sup>; the resulting catabolism of haem may trigger increased membranous HCP-1 expression to compensate. Haem catabolism generates ferrous iron, CO and biliverdin (then reduced to bilirubin)<sup>230</sup>. The  $\text{Fe}^{2+}$  can be processed by the inorganic iron transport chain in the usual manner. It has been demonstrated that induction of HO-1 in Caco-2 cells using  $\text{CdCl}_2$  causes both expression of HCP-1 and haem absorption to be up-regulated<sup>231</sup>.

More recently HCP-1 has been identified as the primary folate transporter in humans, utilising a proton-coupled transport mechanism to import folate from the duodenal lumen. Hereditary folate malabsorption has been shown to be secondary to mutations in the HCP-1 gene<sup>244</sup>.

#### *1.2.3.2 LDL Receptor Related Protein 1 (LRP1)*

Haemopexin (Hx) is a haem scavenger which acts to prevent the toxic inflammatory and pro-oxidant effects of free haem<sup>245-247</sup>. It functions in a way analogous to haptoglobin, which acts as a scavenger; binding to free haemoglobin and enabling uptake by macrophages or splenic monocytes via CD163-mediated endocytosis<sup>248</sup>. It was previously known that a receptor capable of haemopexin-haem uptake through endocytosis existed in hepatocytes, macrophages and syncytiotrophoblasts<sup>249-252</sup>; this receptor was identified as low density lipoprotein receptor-related protein 1<sup>253</sup> using the ligand affinity purification techniques previously utilised to characterize the C163 haptoglobin-haemoglobin receptor<sup>248</sup>.

Binding studies confirmed the ability of LRP1 to bind Hx-haem, whereas the known LRP inhibitor RAP (receptor-associated protein) and anti-LRP IgG both blocked binding<sup>253</sup>.

Experiments on the COS-1 (simian kidney) cell line, which is known to express LRP1, demonstrated uptake of  $^{125}\text{I}$ -Hx-haem and again showed inhibition by RAP/anti-LRP. Furthermore, uptake of  $^{125}\text{I}$ -Hx alone was much less avid than the haem complex<sup>253</sup>. Confocal microscopy demonstrated co-localization of LRP1 and Hx-haem, and also divulged overlap with the early endosomal protein EEA1 (early endosome antigen 1). After 1 hour, the LRP1 had relocated to the cell membrane, whereas the fluorescent Hx-haem remained cytoplasmic; this corresponds exactly with the pattern expected of receptor-mediated endocytosis<sup>253</sup>. Hx-haem and haem were both shown to induce HO-1 mRNA in human LRP- and HO-1-expressing monocytes, with haem alone stimulating the more marked response. HO-1 induction by Hx-haem was reduced by around 50% by RAP and anti-LRP IgG (although not by control IgG)<sup>253</sup>. In combination, these results clearly delineate a role for LRP1 in haem import and also suggest a potential role in the response to inflammation via induction of HO-1. Expression of LRP1 is predominantly in hepatocytes and hepatic and splenic macrophages<sup>254;255</sup>. Unsurprisingly, the majority of exogenous Hx-haem administered by injection can eventually be localized to the liver<sup>256</sup>. The co-localization of LRP1 and HO-1 in the placenta suggests an avenue for iron delivery to the foetus following LRP1-mediated uptake of Hx-haem<sup>257;258</sup>.

#### *1.2.3.3 Feline Leukaemia Virus Subgroup C Receptor (FLVCR)*

FLVCR was first characterized as a receptor for feline leukaemic virus, subgroup C<sup>259;260</sup>. FeLV-C causes aplastic anaemia (red cell aplasia) in cats due to failure of maturation of burst-forming units-erythroid (BFU-E – erythroid progenitors) into colony-forming units-erythroid (CFU-E), thus arresting erythropoiesis<sup>261;262</sup>. FLVCR is, like HCP-1, a member of the major facilitator superfamily<sup>263</sup> and has been shown to comprise 12 hydrophilic transmembrane domains. It is widely expressed in haematopoietic cells, as well as in liver- and intestine-

derived cell lines (all of which tissue types could potentially require a haem-trafficking function). The utility of FLVCR as an exporter of haem has been demonstrated. Initially cells suspected to require the ability to transport haem were shown to exhibit over-expression of FLVCR mRNA and protein. These included the intestinal phenotype Caco-2, hepatic HepG2 and the haematopoietic progenitor line K562. Significantly, FLVCR expression is absent in the mature erythroid line HEL-R which manifests spontaneous haemoglobinization (thus buffering excess intracellular haem)<sup>264</sup>. Engineered over-expression of FLVCR in rat renal epithelial (NRK) cells significantly lowered their haem content, whereas infection of feline embryonic fibroblasts (FEA) with feline leukaemia virus (to block surface expression of FLVCR) led to higher intracellular haem levels<sup>264</sup>. Quantitative fluorescence microscopy using the (fluorescent) haem analogue zinc mesoporphyrin confirmed that NRK and K562 (haematopoietic cells of erythroid phenotype) lines transfected with FLVCR display increased export of haem in a temperature-dependent manner<sup>264</sup>.

FLVCR has also been shown to be integral to normal erythropoiesis. It is avidly expressed by K562 cells; abrogating haem export from these cells with the FeLV ligand caused inhibition of differentiation, and apoptosis when differentiation was induced. CFU-E cells display profuse expression of FLVCR, which is down-regulated as globin synthesis commences and haemoglobinization can occur. Because erythroid cells lack the same regulatory mechanisms for intracellular haem seen in other tissues (via HO-1-mediated degradation etc), FLVCR likely functions as a safety-release valve in CFU-E cells to prevent excess haem causing toxic damage as up-regulation of globin synthesis catches up with that of haem<sup>264</sup>.

#### *1.2.3.4 Breast Cancer Resistance Protein (BCRP)*

BCRP is a member of the ATP-binding cassette (ABC) family (it is also known as ABCG2). These proteins serve as exporters of myriad substrates. BCRP and others of the G subset of

ABC proteins are capable of exporting chemotherapeutic agents from cells, and it was in this context that BCRP was first discovered – conferring resistance to chemotherapy in breast cancer<sup>265;266</sup>. All ABC transporters possess a conserved cytosolic domain, 2 cytosolic nucleotide-binding domains and a minimum of 2 trans-membrane segments. ABCG2 features 6 trans-membrane segments and its nucleotide binding domains are located near the N-terminus of the peptide, in common with all members of the G sub-family<sup>267</sup>.

BCRP is regulated, at least in part, by a hypoxia response element in its promoter region in a similar manner to TfR/IRE. Transcription is thus modulated through binding of HIF1. Akt1 (an upstream regulator of HIF1) directs BCRP expression to the plasma membrane<sup>268</sup>. This localization of BCRP is in contrast to most ABC transporters, which are to be found in cellular organelles<sup>269</sup>. It is this distribution which allows BCRP to function as a detoxifier, exporting drugs, toxins and metabolites from the liver, kidney, intestine and placenta<sup>270;271</sup>.

The ability of BCRP to function as a haem exporter was discovered coincidentally when ABCG2-null mice developed photo-toxicity, subsequently discovered to be secondary to cutaneous deposition of pheophorbide A<sup>272</sup>. This substance, a chlorophyll degradation product found in their diet, is analogous to protoporphyrin IX, which displayed a similar pattern of atypical accumulation in the skin and erythrocytes of ABCG2<sup>-/-</sup> mice. Confirmation of the role of BCRP as a haem exporter was obtained through a number of complimentary experiments. Elevated intracellular levels of protoporphyrin IX were demonstrated in ABCG2<sup>-/-</sup> erythroid progenitors<sup>273</sup>, whereas those over-expressing ABCG2 manifested lower concentrations<sup>274</sup>. ABCG2<sup>-/-</sup> progenitors were demonstrably more susceptible to hypoxia than wild-type cells, although inhibition of ABCG2 in these cells with fumitremorgin C or reserpine rendered them equally sensitive to lower oxygen tensions. Inhibiting haem synthesis in the mutant cells with succinyl acetone prevented intracellular accumulation of porphyrins and enhanced their

survival<sup>275</sup>. In combination, this evidence suggests that ABCG2/BCRP is induced by HIF1 in response to hypoxia to export intracellular porphyrins, thus conferring a survival advantage on haematopoietic cells by minimizing generation of ROS.

#### **1.2.4 Iron and Carcinogenesis**

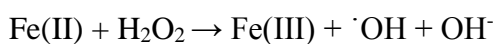
Iron was first linked to carcinogenesis in 1959 by induction of sarcomata in rats injected repeatedly with an iron-dextran complex<sup>276</sup>. These results were replicated in rabbits<sup>277</sup>. Similar effects were subsequently demonstrated in humans receiving therapeutic intramuscular iron injections<sup>278</sup>. Evidence has since continued to mount that iron is fundamentally involved in the pathogenesis of multiple types of cancer in humans.

Initial data supporting this theory was epidemiological. Anaemia appears to be protective against lung cancer in women, while total iron binding capacity is inversely proportional to lung cancer rate<sup>279</sup>. Transferrin binding is significantly higher and total iron binding capacity significantly lower in cancer patients than the normal population (all cancers)<sup>280</sup>. A Chinese study demonstrated that ferritin levels increase and transferrin levels decrease in cancer patients. Carrier status for the hereditary haemochromatosis gene (conferring an iron-overloaded phenotype) is also linked to cancer<sup>279-281</sup>.

A number of potential mechanisms exist through which iron could exert a carcinogenic effect, discussed in detail below.

##### *1.2.4.1 Iron and Redox Chemistry*

A proportion of the carcinogenic effects of iron are dependent on Fenton reaction chemistry. The Fenton reaction describes the production of highly reactive hydroxyl radicals following exposure of ferrous iron to hydrogen peroxide:



These reactive oxygen species (ROS) cause lipid peroxidation and damage to lysosomal membranes, mitochondria and microsomes<sup>282</sup>. Excess iron leads to increasing amounts of ROS via Fenton reaction chemistry. Where iron levels are increased, these carcinogenic effects of oxidative stress could therefore be considered to be due to iron surplus.

A renal carcinoma model demonstrates this concept. Intra-peritoneal injections of Fe-NTA (ferric nitrilotriacetate) induce renal proximal tubular necrosis<sup>283</sup> and eventual renal cell carcinoma<sup>284</sup> in rats and mice. Biochemical and histological analysis confirmed contemporaneous lipid peroxidation in the afflicted rodents<sup>283</sup>. It has thus been argued that induction of oxidative stress by Fe-NTA leads to renal carcinogenesis<sup>285</sup>.

The high incidence of lung cancer in patients exposed to asbestos has been attributed to its high iron content of (around 30% by weight). Asbestos compounds with higher iron content are more likely to be carcinogenic. Evidence suggests that this is due to ROS generated by Fenton reaction chemistry stimulated by iron in the asbestos fibres, although ROS production in macrophage lysosomes and via IL-8 induction is also implicated<sup>285</sup>.

Iron may also exert a carcinogenic effect by inhibiting the protective mechanisms cells employ to defend themselves against oxidative stress. It has been shown to reduce levels of vitamin E and superoxide dismutases, both of which act as reductive systems in times of oxidative stress. However, increasing levels of other reductive substances have been observed in a number of cancers. Glutathione-S-transferase pi (GSTP) is expressed in colon, lung and cervical cancer and was shown to be induced in the renal parenchyma following Fe-NTA administration in the renal cell carcinoma model described above. Higher GSTP levels are associated with poor prognosis in colon cancer. This suggests that certain cancers may have evolved protective mechanisms to survive conditions of persistent oxidative stress<sup>285</sup>.



As well as generating ROS, iron can also cause direct damage to DNA. Ferric citrate is capable of mediating oxidative single- and double-stranded breaks on plasmid DNA in vitro<sup>286</sup>. The majority of the increased free iron in haemochromatosis patients is complexed with citrate<sup>287</sup> and haemochromatosis patients are known to have a much higher incidence of hepatocellular carcinoma than control populations<sup>285</sup>.

#### *1.2.4.2 Iron and Cell Cycling*

Progression of the cell cycle through G<sub>0</sub>, G<sub>1</sub>, S, G<sub>2</sub> and M phases is regulated by cyclins and cyclin-dependent kinases (CDKs). The CDKs act as catalysts for the cyclins; a heterodimer formed of the two comprises the regulatory unit. Cyclins A, D and E are responsible for progress through G<sub>1</sub>, cyclin A for the S phase and cyclin B for the M phase<sup>288</sup>. The regulatory units are in turn controlled by cyclin-activating kinases (CAKs), which phosphorylate the cyclin-CDK complexes to activate them<sup>289</sup>. Inhibitory control of the cycle is mediated via CDK inhibitors (CKIs)<sup>290</sup>.

A key factor in cell cycle control is the p53 tumour suppressor protein, expression of which is up-regulated in response to a variety of insults, including DNA damage, hypoxia, loss of survival signals, disordered cell growth and, critically, iron chelation<sup>291-294</sup>. When any one of these stressors stimulates p53 activation, it is able to arrest the cell or induce apoptosis<sup>295</sup>.

Introducing iron chelators to a variety of cultured cells in vitro reduces expression of cyclins D1, D2, D3, A and B. This phenomenon was observed in cells derived from neuroepitheliomas, breast cancers, leukaemias and Kaposi's sarcoma<sup>296-298</sup>. As the presence of pre-complexed chelators and Fe had no effect, this down-regulation must be due to depletion of available iron by unbound chelator<sup>296</sup>.

Fe chelation reduced expression of cdk2 mRNA<sup>299</sup> in leukaemia cells and the protein<sup>296</sup> in neuroepithelioma cells. Complexes of cyclin D with cdk4, and cyclin E with cdk2, are

necessary for cycling through G<sub>1</sub>/S; when any of these components are scarce, cells may arrest at this stage<sup>296;297</sup>.

The same cell lines demonstrate up-regulation of CKI mRNA in response to iron chelation, particularly p21, although paradoxically this was not mirrored at the protein level<sup>290</sup>.

Iron chelation also induces p53 activity, though this appears to reflect increased activation of latent p53 rather than increased mRNA/protein expression, as both are measurably unchanged<sup>290;296</sup>.

Iron has also been shown to be crucial to the functioning of the enzyme ribonucleotide reductase, the rate-limiting step in DNA synthesis<sup>300-302</sup>. Chelating iron decreases ribonucleotide reductase activity in human leukaemia K562 cells, as well as inhibiting DNA synthesis and cell growth. Similar effects are seen following addition of an anti-TfR antibody to block iron uptake by the cells. These experiments demonstrate the importance of adequate iron in normal ribonucleotide reductase function and thus cell proliferation<sup>303</sup>.

The inhibitory effect of iron chelation on the cycling of malignant cell lines in vitro indicates a crucial role for iron in their propagation, as well as providing a potential therapeutic avenue.

#### *1.2.4.3 Iron and the Immune System*

The anti-tumour effects of macrophages appear to be inhibited by iron; phagocytosed erythrocytes, erythrocyte lysate, haemoglobin, iron dextran and iron salts all diminish the tumouricidal action of macrophages, an effect not achieved by erythrocyte ghost membranes, latex spheres, myoglobin or dextran alone<sup>304</sup>. Excessive iron has also been shown to inhibit function of helper (CD4) T lymphocytes<sup>305;306</sup>, as well as increasing the numbers of active suppressor (CD8) T cells<sup>306</sup>. An increased CD8/CD4 ratio could impair immune surveillance of cancer cells<sup>307</sup>.

#### *1.2.4.4 Iron and Colon Cancer*

As most dietary iron is excreted rather than absorbed, the human colon contains large amounts of iron. As a result a significant amount of work has been done in an effort to link iron and colorectal cancer (CRC). Positive associations have been demonstrated between both dietary iron content and body iron stores and CRC<sup>280;308</sup> and precancerous lesions<sup>309;310</sup>; 75% of large studies in a comprehensive analysis indicated this to be the case<sup>311</sup>. CRC is also more likely in haemochromatosis patients with high intracellular iron levels<sup>312</sup>.

Rodent studies demonstrated that excess dietary iron bestows a more unstable environment on cells of the colonic mucosa. Increasing dietary iron was shown to increase the number of free radicals in the colon, and the amount of subsequent lipid peroxidation<sup>313</sup>, as well as increased numbers of aberrant crypt foci, which are recognised as a pre-malignant change<sup>314</sup>. Rats fed on haem exhibited up-regulated proliferation of colonic epithelium compared to controls (as manifest by increased epithelial uptake of <sup>3</sup>H-thymidine into colonic mucosa)<sup>315</sup>.

When reactive oxygen species degrade lipids, one of the by-products is malondialdehyde<sup>316</sup> – a reactive aldehyde that can be used as a marker for oxidative stress<sup>317</sup>. In HFE-knockout mice (which by definition display a haemochromatosis phenotype), increasing amounts of dietary iron are shown to produce increasing concentrations of colonic and mammary malondialdehyde, implying a more severe degree of damage to the tissues secondary to iron intake<sup>318</sup>.

It has been demonstrated that expression of the proteins concerned with transporting non-haem iron is dysregulated in CRC cells, allowing them to sequester abnormally high levels of intracellular iron. Expression of Dcytb, DMT1 and TfR1 is increased to boost iron import. Although ferroportin expression also increases, it is localised to the intracellular compartment rather than the basement membrane. As hephaestin expression is decreased, the overall effect

is to block iron export and dramatically increase intracellular iron levels<sup>319</sup>. The changes seen in ferroportin expression can be partly explained by the discovery of hepcidin expression in 34% of colorectal cancer specimens. In these cases it was associated with ferroportin inhibition. Urinary hepcidin levels show a positive correlation with pathological T stage of colorectal cancer<sup>320</sup>. High serum ferritin concentrations are linked to a predisposition for developing colonic adenomas<sup>321</sup>.

The potentially carcinogenic effect of rising intracellular iron concentrations in the colonic epithelium has been demonstrated by artificially increasing the amount of iron contained within human intestinal (Caco-2) cells in culture. This led to increased oxidative stress and consequential damage to protein and DNA<sup>322</sup>.

APC is a tumour suppressor gene, mutations in which are strongly implicated in colorectal carcinogenesis. It has been shown that iron is capable of inducing the oncogenic Wnt signalling pathway in cells lacking functional APC, stabilising  $\beta$ -catenin to cause increased expression of mRNA for c-myc and Nkd1, as well as increased cellular proliferation. Transfection of wild-type APC into the cells blocked this effect. Iron excess could therefore help drive the carcinogenic process in APC-deficient cells<sup>323</sup>.

The role for iron in colorectal carcinogenesis is thus clear, with both a demonstrable mechanism for increasing iron levels within colorectal cancer cells, and a tangible oncogenic effect of iron in APC deficient colonocytes.

#### *1.2.4.5 Iron and Hepatocellular Carcinoma*

Hepatocellular carcinoma (HCC) is usually the endpoint of a pathological progression of parenchymal liver disease. Some form of initial, usually chronic, physiological insult to hepatocytes results in cellular damage and death. Fibrosis and scarring ensue, followed by regeneration of the damaged liver<sup>324</sup>. Repeated cycles of destruction and regeneration lead to

the development of cirrhosis, which confers a 200-fold increase in the risk of developing HCC<sup>325</sup>.

There are myriad aetiological factors recognised in the pathogenesis of cirrhosis. The most common in the Western world are alcohol and hepatitis viruses B and C. However, iron is also implicated, primarily in patients suffering from hereditary haemochromatosis<sup>326</sup>. HCC is in fact the leading cause of death in haemochromatosis patients<sup>327</sup>. Classically, haemochromatosis was considered to arise from one of two point mutations in the HFE gene (juvenile haemochromatosis, discussed in relation to hepcidin in section 1.2.2.6, represents a different disease phenotype). Under normal circumstances, HFE will complex with TfR at the cell membrane of hepatocytes in order to inhibit transferrin binding and therefore iron import. This action relies on the ability of HFE to bind to  $\beta_2$ -microglobulin; an ability abrogated by the C282Y mutation which substitutes a tyrosine residue for one of 4 invariant cysteine residues. These 4 residues are a shared feature with major histocompatibility complex (MHC) class I molecules, in which they form disulphide bridges in the  $\alpha_2$  and  $\alpha_3$  domains<sup>328</sup>. In fact, HFE was initially known as HLA-H, owing to the marked similarity between the two proteins<sup>326</sup>. Around 90% of haemochromatosis patients are homozygous for the C282Y mutation<sup>329</sup>, which also localises HFE to the cytosol rather than the cell membrane where wild-type HFE is expressed<sup>330;331</sup>. This autosomal recessive inheritance pattern is in contrast to those described in relation to the ferroportin mutations discussed earlier, which also instigate a haemochromatosis phenotype<sup>155;156</sup>.

A second point mutation has also been described in “classical” haemochromatosis; His63→Asp (H63D) is frequently observed in patients heterozygous for the C282Y mutation. Although the precise nature of the dysfunction prompted in HFE by this mutation is unclear, it

is thought to relate to changes in the way in which HFE is able to interact with other proteins<sup>329</sup>.

Whichever of the HFE mutations is responsible, the consequences of haemochromatosis are unaltered. Increased binding affinity between transferrin and TfR results in increased cellular uptake of iron in all tissues, but particularly the liver, which is the primary organ involved in iron storage and metabolism<sup>326</sup>. Iron then mediates cellular and DNA damage to initiate the sequence of events culminating in development of neoplasia.

It is worth noting that iron plays a secondary role in HCC pathogenesis through its function as a cofactor for viral propagation. Persistent infection with hepatitis B or C also results in a chronic insult to hepatocytes that may eventually lead to cirrhosis and hepatoma<sup>332</sup>.

Also of interest is the fact that liver iron concentrations are slightly raised in HCC even when haemochromatosis and cirrhosis have no part in the disease process<sup>333</sup>.

#### *1.2.4.6 Iron and Renal Cell Carcinoma*

The link between iron and renal cell carcinoma (as well as other types of renal malignancy) is less well understood than that for CRC and HCC.

Rodent studies have demonstrated the carcinogenic properties of ferric nitrilotriacetate (Fe-NTA) on the kidney<sup>334</sup>. Oxidative damage to the renal tubules causes acute tubular necrosis; repeated exposure may eventually lead to RCC in a similar fashion to the development of HCC in cirrhotic livers. Products of lipid peroxidation, including malondialdehyde, were found to be elevated in treated rats<sup>335</sup>. Fe-NTA was a more potent carcinogen than its cupric equivalent, and NTA alone had no carcinogenic effects<sup>336</sup>. The p16 tumour suppressor gene suffered allelic loss after treatment with Fe-NTA, providing a second potential aetiopathogenic mechanism for iron-induced RCC in rats<sup>337</sup>.

In terms of the human disease, population studies provide the only hard evidence that iron may be an environmental carcinogen affecting the kidney. In one such study, involving multiple international centres, the relative risk of RCC was found to be 1.6 in iron and steel workers compared to normal individuals (95% confidence interval 1.2-2.2)<sup>338</sup>. However, no specific compounds have been identified and no particular genetic mutations discovered.

#### *1.2.4.7 Iron and Other (Non-Mammary) Cancers*

Increased incidence of both lung and stomach cancer has been reported in iron and steel workers<sup>339;340</sup>. It is thought that the responsible agent is the dust circulating in these environments, which is either ingested or inhaled. These dusts display surface redox activity in aqueous solution<sup>341;342</sup> and have been shown to be increasingly reactive when a larger fraction of the dust is formed of bio-available iron. The redox activity of similar dusts found in coal and asbestos-handling facilities was found to be dependent on available free iron and was also proportional to ferritin induction and lipid peroxidation in cells exposed to them<sup>343-347</sup>.

Iron seems to act as a potentiator of chemically-induced skin cancer in murine models. Using 7,12-dimethylbenz(a)anthracene to induce murine skin cancer was more effective in conjunction with intramuscular iron injections<sup>348</sup>. A low iron diet led to a fall in the incidence of tumour formation and down-regulated the rate of conversion of papillomas to cancers<sup>349</sup>.

Iron supplementation has also been seen to stimulate proliferation, inflammation, nitric oxide synthase and tumour formation in rats with Barrett's oesophagus<sup>350</sup>.

#### *1.2.4.8 Iron and Breast Cancer*

Iron has long been proposed by some groups to be a potential carcinogen responsible for the development of breast cancer. It has been demonstrated that iron (in common with other transition metals capable of propagating oxidative stress) is present in higher than expected

concentrations in malignant human breast tissue when compared to normal<sup>351</sup>. In opposition to this argument, a number of studies have failed to demonstrate any significant relationship between dietary intake of iron (whether haem or non-haem) and breast cancer risk<sup>352-354</sup>. However, it has been shown that plasma concentrations of malondialdehyde are elevated in breast cancer<sup>354;355</sup>, suggesting a role for oxidative stress, which is known to be induced by increased levels of free iron<sup>104;105</sup>. Research has also revealed increased  $\cdot$ OH-DNA adducts and single/double strand DNA breaks in patients with invasive breast cancer, consistent with damage due to oxidative stress<sup>356-358</sup>. Additionally, it has been demonstrated that there is active Wnt signalling in breast cancer, with stabilization of  $\beta$ -catenin being observed in human breast tumours, and that induction of Wnt signalling causes murine mammary carcinogenesis<sup>359</sup>. It is conceivable that dysregulation in the iron transport chain could confer upon breast cancer cells the ability to sequester unusually large amounts of intra-cellular iron, even in the face of normal circulating concentrations.

Ferritin levels in malignant breast tissue have been shown to be 6 times higher than in normal tissue, although the tissue samples were unmatched (taken from different individuals)<sup>360</sup>. In addition, ferritin concentration corresponds to the degree of proliferation of malignant breast epithelium and the extent of epithelial pleomorphism<sup>360</sup>.

More recent work has demonstrated that ferritin is over-expressed in aggressive breast cancer cell lines of mesenchymal phenotype (such as MDA-MB-231) compared to more indolent lines of epithelial phenotype (MCF7, T47D) in tissue culture<sup>361</sup>. The distribution of ferritin within cells also changes; the more aggressive lines exhibit higher expression in the cytoplasmic and chromatin-bound nuclear fractions<sup>361</sup>. It has previously been demonstrated that heavy chain ferritin can translocate to cell nuclei to protect DNA in the face of iron-overload<sup>362</sup> – breast cancer lines may have evolved to utilise a similar survival strategy.



Increased ferritin levels have also been shown to correlate with shrinkage of the labile iron pool, which is thought to trigger induction of vaso-active endothelial growth factor (VEGF) and bestow a more aggressive phenotype<sup>363</sup>. These observations tally with clinical studies demonstrating a link between tissue concentrations of H-ferritin mRNA and both clinical and histological prognosis in breast cancer, including nodal status, the presence of metastases and overall clinical staging<sup>364</sup>.

It is not unreasonable to suppose that, although an increase in the labile iron pool may act as an initiator or propagator of malignancy via increased oxidative stress, the more malignant breast cancers could potentially achieve higher rates of survival and proliferation through an increase in ferritin expression following phenotypic transformation. This would mitigate the effects of increased iron import by buffering the larger labile iron pool, protecting cell structures and DNA from oxidative damage<sup>361</sup>. Similar mechanisms have already been reported in erythroid, leukaemic and hepatic cell lines<sup>136;137;365</sup>.

It has been shown that ferroportin plays a role in breast cancer – in particular that ferroportin levels are reduced in cancer relative to normal tissue. This is true both in vitro and in human tissue. Cultured epithelial breast cells display expression of ferroportin, which can be regulated by hepcidin<sup>366</sup>. Ferroportin levels are reduced in malignant cell lines<sup>366</sup>. There is an inversely proportional relationship between ferroportin levels and the degree of anaplasia observed in human cancer specimens, where lower ferroportin levels are seen in the most anaplastic specimens<sup>366</sup>. Xenograft models have demonstrated that transfection of breast cancer cells with ferroportin significantly inhibits development of breast cancers in the mouse mammary fat pad after orthotopic implantation<sup>366</sup>. Gene expression profiling has revealed that decreased ferroportin expression is an independent predictor of poor prognosis in terms of disease- and metastasis-free survival<sup>366</sup>. Ferroportin down-regulation has been found to

promote breast cancer growth<sup>367</sup> and this has recently been attributed to decreased expression of the transcription factors Nrf2 and MZF-1<sup>368</sup>.

Transferrin receptor expression is known to be altered in breast cancer. TfR expression has been demonstrated to be up-regulated in breast cancer relative to normal tissue<sup>369;370</sup>. Immunohistochemical investigation has exposed a negative association between TfR expression and the degree of differentiation of breast tumours (high TfR correlated with poorly differentiated cancers)<sup>371</sup> while rodent studies revealed TfR to confer on breast cancer cell lines an increased capability to proliferate and metastasize<sup>372</sup>. High levels of expression of TfR mRNA correspond to poorly differentiated tumours<sup>364</sup> and gene expression studies demonstrate high TfR gene expression to be a predictor of poor prognosis in breast cancer<sup>367</sup>.

Recent in vitro studies demonstrated increased expression of both DMT1 and TfR in human breast cancer cells compared to normal breast epithelium, and also showed absent ferroportin expression in the cancer cells<sup>373</sup>. Proliferation of 4T1 murine mammary adenocarcinoma cells in culture is inhibited by TfR antisense oligonucleotides, which are also shown to inhibit TfR mRNA expression and intracellular iron levels. The same oligonucleotides inhibited tumour and metastasis growth in the 4T1 murine mammary adenocarcinoma model<sup>373</sup>.

IRP2 over-expression has recently been linked to more aggressive breast carcinomas, the effect being attributed to a combination of up-regulated TfR1 expression and inhibition of ferritin H (the overall effect being to increase the intra-cellular labile iron pool)<sup>374</sup>.

The above evidence suggests that non-haem iron transport is significantly altered in breast cancer and that the expression profile of the transport proteins is modified to sequester large amounts of intracellular iron. Whether this is simply a mechanism to acquire the iron needed to meet the demands of a rapidly proliferating cell population, or a carcinogenic process is unclear. No evidence supports similar dysregulation in haem transport pathways.

### 1.2.5 Iron Chelators

Given that breast cancer, in common with many other malignancies, displays a phenotype specifically geared towards acquisition of iron<sup>360;366;367;369;370</sup>, it is not unreasonable to suppose that agents designed to deprive cancer cells of iron could potentially be useful anti-tumour therapies. Binding Fe<sup>3+</sup> prevents Fenton reaction chemistry and limits oxidative stress. Minimising the available iron for use in cell cycling and proliferation will also preferentially inhibit malignant cell populations with a higher turnover than normal cells.

Chelators are compounds that are capable of binding free metal ions. Iron chelators were first utilised medically in the treatment of conditions such as  $\beta$ -thalassaemia, which involve iron toxicity. As the role of iron in carcinogenesis has become better understood, the potential of chelators to inhibit cancer progression or even cause tumours to go into remission has been investigated.

Desferrioxamine (DFO) was the first chelating agent used against  $\beta$ -thalassaemia<sup>375</sup>. It has since been shown to possess potent anti-cancer activity in vitro, inhibiting proliferation and inducing apoptosis in malignant cell lines including cervical and ovarian cancer, and more recently breast cancers<sup>376-378</sup>. In tissue culture DFO has been shown to potentiate the effects of traditional chemotherapy agents<sup>379</sup>. However a short half life and the lack of an orally active preparation, along with significant side effects and limited efficacy in many trials, make widespread use as an anti-cancer medication unfeasible<sup>380</sup>.

Deferiprone has a similar spectrum of activity in vitro against malignant cell lines including Kaposi's sarcoma<sup>298</sup> and (oral) squamous cell carcinoma<sup>381</sup>. It has the advantage that it is available as an oral preparation but in vivo studies did not report significant anti-cancer activity and the side effect profile again limits frequent use as a therapeutic agent<sup>380</sup>.

Deferasirox (ICL670A) is a chelating agent frequently used in treating iron overload. It has demonstrable anti-tumour effects against leukaemia and hepatoma cell lines in vivo<sup>382;383</sup>. The anti-hepatoma activity is more marked than the inhibition of proliferation and viability exerted on normal hepatocytes, implying a degree of selectivity for malignant populations<sup>382</sup>. Deferasirox has proven anti-tumour activity in vivo, suppressing leukaemia development in a murine model<sup>383</sup>.

Although it has been demonstrated that depriving MCF-7 cells of iron in vitro causes apoptosis, and that the effect was replicated in a rat mammary adenocarcinoma model<sup>384</sup>, there has been no other meaningful investigation of the wider effects of iron chelation on breast carcinoma, or any comparison of the relative effects of chelation on malignant compared to normal breast tissue.

## **1.3 Hypothesis**

### **Background**

A role for iron in the development and propagation of a number of cancers, including breast, has now been established.

Although there are defined oncogenic mechanisms through which iron induces carcinogenesis in certain organs (notably the colon), it is not yet clear whether the demonstrable changes in iron transport observed in breast cancer are a response to the demand for iron in rapidly proliferating tissue or a fundamental requirement for breast carcinogenesis.

Furthermore, although clear in vitro and in vivo evidence describes changes in expression of many of the proteins involved in transporting non-haem iron in cancer compared to normal breast tissue, no studies compared expression in prospectively collected matched tissue pairs, and no evidence yet suggests a role for dysregulated haem transport in breast cancer.

Finally, despite mounting evidence advocating iron chelation in the treatment of a number of cancers, there has been very little evaluation of the effects of iron chelators in breast cancer.

### **Hypothesis**

The malignant progression of breast cancer is associated with modulations in expression of both inorganic and haem iron metabolic machinery. These modulations confer a selective advantage to breast cells in acquiring increased levels of inorganic and haem iron and this in turn stimulates development of a more aggressive phenotype.

Suppression of cellular iron through the use of chelators will depress the aggressive behaviour of these cells.

## **1.4 Aims**

The aims of this study are:

1. Examine the expression of non-haem iron transport proteins in breast carcinoma compared to normal breast tissue and validate these results using prospectively collected matched tissue pairs.
2. Evaluate expression of haem transport proteins in breast carcinoma compared to normal breast tissue.
3. Determine the effects of iron and haem on the phenotype of benign and malignant human mammary cells in vitro.
4. Determine the effects of iron and haem on expression of their respective transport proteins in benign and malignant human mammary cells in vitro.
5. Investigate the effects of iron chelation on cell phenotype and expression of iron transport proteins.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 Materials**

#### **2.1.1 Laboratory Reagents**

General, cell culture and molecular biology reagents were obtained from:

*Anatomical Pathology International, Runcorn, Cheshire*

Shandon Consul-Mount

*BD Biosciences, Bedford, Bedfordshire*

Matrigel (basement membrane matrix)

*Bioline Ltd, London*

Sensimix (PCR mastermix)

*Dako, Ely, Cambridgeshire*

Delimiting pen; EnVision<sup>TM</sup> Detection System; REAL antibody diluent

*Eurogentec, Fawley, Hampshire*

18S rRNA control kit (FAM-TAMRA); 5'-FAM, 3'-TAMRA double dye oligonucleotides;  
Reverse transcriptase core kit; qPCR optical seals; Sub-skirted, low profile 96-well qPCR  
plates

***Fisher Scientific, Loughborough, Leics***

BCA protein assay kit (Pierce); Ethanol; Methanol; Mr Frosty cryobox (Nalgene); Xylene

***GE Healthcare UK Ltd, Little Chalfont, Bucks***

Amersham ECL Western blotting detection reagents; Amersham full-range rainbow molecular weight markers; Amersham Hyobond-P PVDF Membrane; Amersham hyperfilm ECL

***Invitrogen Ltd, Paisley, Renfrewshire (incorporating Gibco BRL)***

Dulbecco's Modified Eagle's Medium (DMEM); DMEM x2; Histostain Plus immunohistochemistry kit; NP40 cell lysis buffer; PenStrep (5000U/ml Penicillin, 5000µg/ml Streptomycin); Trypsin/EDTAx4Na

***Leica Microsystems (UK) Ltd, Milton Keynes***

W-Cap antigen retrieval solution

***Life Technologies Ltd, Inchinnan Business Park, Paisley***

Combined probe and primer PCR kits

***PAA Laboratories, Yeovil, Somerset***

Standard quality fetal bovine serum

***Roche Diagnostics Ltd, Burgess Hill, West Sussex***

BrdU cell proliferation ELISA kit (colorimetric)



***Sigma-Aldrich Company Limited, Poole, Dorset***

Agar; Alamar blue; Ammonium Persulfate;  $\beta$ -mercaptoethanol; Bovine serum albumin; Bromophenol blue; Chloroform; Dimethyl sulphoxide; Ferric Chloride; Ferrous sulphate heptahydrate; Ferrozine; Fibronectin adhesion-promoting peptide; Glycine; Guanidine hydrochloride; Hemin; HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); Hydrochloric acid; Hydrogen peroxide; Isopropanol; Mayer's haematoxylin 0.1% solution; 4-Morpholinepropanesulfonic acid (MOPS); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); Nuclease-free water; Phosphate buffered saline tablets; Polyacrylamide; Propidium iodide; RNAlater stabilization solution; RNase A; Sodium acetate; Sodium ascorbate; Sodium chloride; Sodium citrate; Sodium deoxycholate; Sodium dodecyl sulphate (SDS); Sodium hydroxide; Sodium pyrophosphate; Trichloroacetic acid; Tris-HCl; Trizma base; N,N,N',N'-Tetramethylethylenediamine (TEMED); Trizol reagent

### 2.1.2 Primary Antibodies

Antigen	Antibody Type	Product Number
<i>Abcam, Cambridge Science Park, Cambridge</i>		
$\beta$ -actin	Mouse monoclonal IgG1	ab8226
BCRP	Mouse monoclonal IgG2a	ab3380
Ferritin	Rabbit polyclonal IgG	ab65080
Ferroportin	Rabbit polyclonal IgG	ab58695
FLVCR	Mouse monoclonal IgG2b	ab57317
HCP1	Rabbit polyclonal IgG	ab25134
Hepcidin	Rabbit polyclonal IgG	ab30760
Hephaestin	Mouse monoclonal IgG2a	ab56729
LRP1	Mouse monoclonal IgG1	ab20384
<i>Invitrogen (Zymed), Inchinnan Business Park, Paisley</i>		
Transferrin Receptor 1	Mouse monoclonal IgG1	13-6800

**Table 2-1 Primary Antibodies**

#### *Gifts*

Dcytb; rabbit polyclonal IgG, clone 834 (kind gift from Professor A McKie)

DMT1; rabbit polyclonal (kind gift from Professor G Anderson)

#### **Secondary antibodies**

##### *Amersham Biosciences, Amersham, Bucks*

Horseradish peroxidase-conjugated sheep anti-mouse IgG

Horseradish peroxidase-conjugated donkey anti-rabbit IgG

<b>Primary Antibodies</b>	
<b>Target Antigen</b>	<b>Optimal Dilution</b>
β-actin	WB: 1/1000
BCRP	IHC: 1/20 WB: 1/500
Dcytb	IHC: 1/250 WB: 1/1000
DMT-1	IHC: 1/250 WB: 1/400
Ferritin	IHC: 1/400 WB: 1/2500
Ferroportin	IHC: 1/100 WB: 1/2000
FLVCR	IHC: 1/100 WB: 1/500
HCP-1	IHC: 1/50 WB: 1/250
Hepcidin	IHC: 1/50 WB: 1/1000
Hephaestin	IHC: 1/100 WB: 1/200
LRP	WB: 1/500
TfR1	IHC: 1/400 WB: 1/1000
<b>Secondary Antibodies</b>	
<b>Antibody</b>	<b>Optimal Dilution</b>
Mouse IgG-HRP	1/10000
Rabbit IgG-HRP	1/10000
Envision Rabbit/Mouse (HRP)	1/10000

**Table 2-2 Primary and Secondary Antibody Concentrations**

*IHC – Immunohistochemistry; WB – Western Blotting*

### 2.1.3 Oligonucleotide Sequences

Probes and primers were designed against human DNA sequences and obtained from Eurogentec. In addition, assays for BCRP and FLVCR mRNA utilised pre-inventoried TaqMan gene expression assay kits, combining probes and primers. These kits were obtained from Life Technologies Ltd.

Gene	Probe	Forward Primer	Reverse Primer
Dcytb	CCAGGGCATCGCCATCATC GT	CATGGTCACCGGC TTCGT	CAGGTCCACGGCAGT CTGTA
DMT1	AGGCAGCTCCACACTGTGA ACTAAAATCATA	CATGAGGAAGAA GCAGCTGAAGA	TG TTCAGGACCCAGC ACCAT
Ferritin	AGTTGTATGCCTCCTACGTC ATCTGTCTATGTCTTGTTAT	GGAACATGCTGAG AAACTGATGAA	CATCACAGTCTGGTT TCTTGATATCC
Ferroportin	AGGATTGACCAGTTAACCA ACATCTTAGCCC	AGCAAATATGAAT GCCACAATACG	CAAATGTCATAATCT GGCCAACAG
HCP1	ATGGCTTGCTTTTCCTCTCA TTAGTCATCACA	CTATCACGCCTCT CATGTTAC	GGAGAGTTTAGCCCG GATGAC
Hepcidin	AGCTGCAACCCCAGG	CCCACAACAGACG GGACAA	TCTGGAACATGGGCA TCC
Hephaestin	ACAGTGACATAGTGGCTTC CAGCTTCTTAAAGTCTG	GGAAGAAATGTCA TCACGAAC	TCCCCCTATCCGGTT CTTG
IRP1	ACTCCTATGGCTCCCGCCG AGG	CCTGCCTAACTCC ACGAGAATT	TGCCATGACGGCGTC AT
IRP2	TTTGACAAACAGAGGCCTT ACCCC	AGGAATAGTGCTG CCGCTAAG	TCGAGCTCCGTAAGA GTTGAATT
LRP1	TGCCATTTACTCAGCCCGTT ACGACG	TGGATTGACGCCA GGTCAG	CCCGAAGCACCTCCA TGT
TfR1	AAAGACAGCGCTCAAACT CGGTGATCATAG	CGTGATCAACATT TTGTTAAGATTCA	CCACATAACCCCCAG GATTCT

**Table 2-3 Probe and Primer Base Sequences**

#### 2.1.4 Cell Lines

All cell lines used were human mammary lines. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, 110mg/l sodium pyruvate and 4.5g/l glucose, supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) Penicillin/Streptomycin solution (5000IU/ml Penicillin, 5000µg/ml Streptomycin).

Cell Line	Phenotype	Growth Medium
HB2	Normal epithelium <sup>385</sup>	DMEM
MCF7	Malignant (adenocarcinoma) <sup>386</sup>	DMEM
MDA-MB-231	Malignant (adenocarcinoma) <sup>387</sup>	DMEM

**Table 2-4 Cell Lines Used**

### **2.1.5 Fresh Tissue**

Fresh tissue was obtained from the Queen Elizabeth Hospital and City Hospital, Birmingham (UK). Informed consent was obtained by a trained member of the laboratory team using forms approved by the ethics committee. Material was taken from mastectomy specimens only. Donor patients were naïve to both hormone and chemotherapy to ensure no modification of cancer phenotype or protein expression. Fresh specimens were taken to a consultant pathologist to remove samples of tumour and matched normal tissue (one normal sample from the extremity of each quadrant of the breast; hence wide local excision specimens were unusable). Only samples confirmed as normal following formal histological assessment of the entire specimen by the pathologist were used as matched normal tissue for analysis. At Queen Elizabeth hospital the samples were immediately flash frozen in liquid nitrogen and stored at -80°C until needed. On the City Hospital site, samples were kept at 4°C in RNAlater solution (which preserves both protein and RNA) until they could be transported to the laboratory for storage at -80°C (RNAlater having first been removed to avoid crystal formation). A total of 20 samples were collected during this study, 19 of which were obtained from the City Hospital site using the RNAlater protocol.

## **2.2 Methods**

### **2.2.1 Ethical Approval**

Ethical approval for this study was sought from the Coventry Research Ethics Committee and granted on April 9<sup>th</sup> 2008 (REC reference number 08/H1210/11). Site specific approval was granted for tissue collection from Birmingham's Queen Elizabeth and City hospitals and confirmed by local R+D departments within University Hospital Birmingham and Sandwell and West Birmingham Hospitals NHS Trusts respectively.

### **2.2.2 Immunohistochemistry**

#### *2.2.2.1 Immunohistochemistry Using W-Cap*

W-Cap is a mixture of retrieval salts and paraffin sequestering agents. It was used in conjunction with the Dako REAL EnVision detection system. W-Cap was pre-heated to 95°C in a water bath. As it heated, formalin-fixed paraffin-embedded tissue sections were warmed in an incubator at 60°C to soften the wax. Warmed slides were bathed in heated W-Cap for 1 hour then allowed to cool to 40°C. After a wash in warm tap water, then TBS at pH 7.6, slides were blotted dry and sections outlined with a Dako delimiting pen. Primary antibodies were diluted in Dako diluent to appropriate concentrations (determined by previous optimization – see table 2-1). 100µl antibody solution (more if the tissue was not adequately covered) was pipetted onto each section and left overnight at 4°C in humidified chambers. Primary antibody was then removed and sections washed twice for 5 minutes in TBS. Samples were incubated with 100µl EnVision reagent (peroxidase-conjugated dextran coupled with goat anti-rabbit/mouse immunoglobulins) for 30 minutes. Following 2 further 5 minute washes in TBS, 100µl of 1:50 DAB+ chromogen in substrate buffer was left on the sections for 8-10 minutes

to visualise immuno-reactivity. Sections were washed in running water then left for 1 minute in 0.1% (v/v) Mayer's haematoxylin solution. A further running water wash was followed by 30 seconds in warm tap water to blue. Sections were then passed through 2 ethanol baths and 2 xylene baths for 5 minutes each and were mounted with cover-slips using Shandon Consul-Mount. Sections were left (flat) overnight in the fume cupboard to dry fully before scoring.

#### *2.2.2.2 Immunohistochemistry Using Citric Acid*

Sections were passed through 2 xylene baths to de-wax then dehydrated in 2 ethanol baths (5 minutes each). There followed a 20 minute immersion in 10% (v/v) hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. For antigen retrieval the sections were then immersed in 0.01M tri-sodium citrate buffer at pH 6.0 and microwaved for 5 minutes 3 times, topping up the buffer in between. After 5 minutes cooling, sections were washed in TBS and the process continued with the Dako system as described above.

#### *2.2.2.3 Evaluation of Immunostaining*

Sections prepared as described above were examined using a Nikon Eclipse E600 microscope. Images were taken with a Nikon DXM1200F digital camera and Nikon ACT-1 (version 2.62) software. Cellular localisation of antigen was documented (nuclear, cytoplasmic or cell surface) and staining intensity (indicating antigen expression) graded from 0 (absent), through 1 (weak) and 2 (moderate), to 3 (strong). Evaluation of immunostaining was performed by the author and validated by an independent consultant histopathologist. Positive (tissue known to express the relevant antigen) and negative (primary antibody not included) controls were included with each series of processed slides. Each series consisted of 5 normal breast specimens, 5 DCIS and 20 breast cancer.



### 2.2.3 Cell Culture

#### 2.2.3.1 Routine Cell Culture

Appropriate growth media for the cell lines investigated are listed in table 2-4. The additives present in the basic media are described in section 2.2.3.2. Unless specified in individual protocols, all media and trypsin were pre-warmed to 37°C in a water bath before being used on cells. A laminar flow tissue culture cabinet and full aseptic technique were employed for all cell culture procedures. Cells were passaged through 25cm<sup>3</sup> and 75cm<sup>3</sup> tissue culture flasks and bulked/maintained in 150cm<sup>3</sup> flasks. Once 90-95% confluence was reached, medium was removed by aspiration. Cells were washed in (calcium- and magnesium-free) phosphate-buffered saline (PBS) and lysed by incubating at 37°C in a solution containing 0.05% trypsin and 0.53mM EDTAx4Na. Once detached, an equal volume of culture medium was added to neutralise the trypsin. The suspension was aspirated into an appropriately sized centrifuge tube and centrifuged for 5 minutes at 1500rpm. The supernatant was aspirated and the pellet re-suspended in either normal medium, for splitting into further culture flasks, or a freezing solution made with 9 parts FCS to 1 part DMSO. Once cells were suspended in an appropriate volume of freezing solution, 1ml aliquots were pipetted into cryovials. These were placed in a Mr Frosty® freezing container (Nalgene) in the -80°C freezer for 5 days then transferred to liquid nitrogen for long term storage. The Mr Frosty® slows the rate of freezing to a maximum of 1°C/min, allowing effective cryopreservation.

To resurrect the cells, the cryovials were rapidly warmed to 37°C and the cells re-suspended in culture medium in a 25cm<sup>2</sup> culture flask. The medium was changed the following day whether or not the cells required further splitting.

### 2.2.3.2 Cell Line Stimulation

Standard formulations for iron- and haem-containing media are shown below in table 2-5. A range of concentrations were used during initial proliferation and viability assays to determine optimal stimulation conditions for each cell line. FCS has been shown to have an iron content of approximately 30µmol/L<sup>432</sup>. Therefore, although control media will have contained a very small amount of iron, concentration in the experimental media was supra-physiological by a factor of several thousand. Valid comparisons could thus be made and appropriate conclusions drawn.

Medium	Basic Medium (DMEM, 10%FCS)	Ferrous Sulphate (100mM)	Sodium Ascorbate (100mM)	Hemin (1mM)
Control	50ml	-	5µl	-
IL (100µM)	50ml	50µl	5µl	-
HL (40µM)	50ml	-	-	2ml

**Table 2-5: Basic Cell Stimulation Media**

*IL – Iron Loaded; HL – Haem Loaded*

**Ferrous Sulphate:** Filter sterilised distilled water was used to prepare stock solutions of 100mM ferrous sulphate and 100mM sodium ascorbate (which aids iron absorption<sup>388</sup>). These solutions were refrigerated at 4°C in the dark until needed.

**Haem Iron:** A 2mM haem solution was prepared with filter sterilised distilled water and solubilised with an equal volume of 0.1mM sodium hydroxide (giving a final concentration of 1mM). Hydrochloric acid was added drop-wise to neutralise pH to 7.4. The solution was kept refrigerated in the dark until needed.

## 2.2.4 Determination of Intracellular Iron Content

### 2.2.4.1 Ferrozine Assay

The ferrozine assay quantifies intracellular iron levels<sup>389</sup>. Cells were cultured in triplicate in 6 well plates with appropriate media. After incubation they were washed 3 times with 1ml sterile PBS (to remove excess iron-containing medium) and trypsinized. They were then moved into a microcentrifuge tube and spun for 5 minutes at 12000rpm. The trypsin was aspirated and the pellet re-suspended in 100µl HEPES saline (10mM HEPES in 0.9% (w/v) NaCl at pH 7.4). 90µl of this suspension was removed and added to 200µl 20% (w/v) TCA in 4% (w/v) sodium pyrophosphate (5µl of the remainder was added to 95µl RIPA solution and used for protein quantification – see section 2.2.4.2). This was boiled for 5 minutes then re-centrifuged for a further 5 minutes at 12000rpm. 200µl of the supernatant was aspirated and added to 100µl 0.23M sodium ascorbate, 80µl 10mM ferrozine and 420µl 2M sodium acetate. This was thoroughly mixed and 200µl aliquots placed in triplicate in a flat-bottomed 96-well plate. A blank (HEPES + TCA + reaction mix) and a standard (the same mixture with 1µl 10mM FeCl<sub>3</sub> added to make a 12.5µM FeCl<sub>3</sub> solution) were included. The colorimetric change in each sample was measured using a Bio-Tek ELx800 plate reader at 550nm. The absorbance in the blank was used to correct each sample before determining iron concentration through comparison with the FeCl<sub>3</sub> control. Experiments were repeated 3 times.

### 2.2.4.2 Protein Assay

A BCA assay was used to determine sample protein concentrations. Standard bovine serum albumin solutions were made up at concentrations ranging from 0-2mg/ml. 20µl of each experimental sample and each standard were placed in triplicate in a flat-bottomed 96-well plate. 200µl BCA working solution (reagent A 50:1 reagent B) was added to each well and the

absorbance measured at 562nm using a Bio-Tek ELx800 plate reader. The protein standards were used to plot optical density against protein concentration using Microsoft Excel software. A best-fit line was used to generate a  $y=mx+c$  equation. If necessary the absorbance of each experimental sample was corrected for dilution, then the equation was used to calculate protein concentration in mg/ml.

## **2.2.5 Cell Phenotype Assays**

### *2.2.5.1 MTT Viability Assay*

MTT contains tetrazolium rings which are cleaved by mitochondrial dehydrogenase. This leads to the formation of purple formazan crystals within cells. MTT can thus be used to quantify viable cell numbers, as only healthy, metabolically active cells express the enzyme<sup>390</sup>. A 5mg/ml stock solution of MTT was made in sterile PBS. Cells were seeded into 96-well plates and cultured for appropriate lengths of time in 100µl experimental growth medium. 8 wells were allocated to each growth medium. Following stimulation, 10µl of MTT solution was added to each well and the plates re-incubated for 3 hours. The medium was then removed by gentle aspiration and replaced with 50µl DMSO. Plates were left for 30 minutes at room temperature then the absorbance of the resulting purple solution at 490nm was measured using a Bio-Tek ELx800 microplate reader. Percentage viability with respect to control was calculated from these optical densities. Experiments were repeated 3 times.

### *2.2.5.2 BrdU Proliferation Assay*

When introduced to cell culture medium, BrdU is incorporated into cellular DNA in place of thymidine during replication. This allows a colorimetric ELISA to quantify cellular proliferation<sup>391</sup>.

Cells were seeded into 96-well plates and cultured for appropriate lengths of time in 100µl experimental growth medium. 8 wells were allocated to each growth medium. A stock solution of 10mM BrdU labelling reagent diluted 1:100 in culture medium was made and 10µl added to each well after stimulation. Included in each assay were a blank to correct for binding to the well itself (culture medium and BrdU, but no cells), and a background control to correct for immunoreactivity between anti-BrdU-peroxidase and unlabelled cells (10µl of blank medium added to the wells). The plates were re-incubated for 4 hours to allow BrdU incorporation then the labelling mixture was removed by gentle aspiration. 100µl FixDenat solution was added to each well and left at room temperature for 30 minutes. FixDenat fixes the cells and denatures DNA to expose incorporated BrdU. It was replaced with 50µl/well 1:100 anti-BrdU-peroxidase antibody solution (diluent supplied with the kit) and left at room temperature for 90 minutes. Following removal of the antibody solution, wells were washed 3 times with 200µl of assay wash solution then left for 5-30 minutes with 50µl/well of TMB substrate solution. TMB is a chromogenic substrate which forms a blue substance when allowed to react with peroxidase enzymes. The density of the blue colouration corresponds to the number of immune complexes formed between incorporated BrdU and anti-BrdU-POD, allowing quantification of cell proliferation. Absorbance of each well at 370nm was measured using a Bio-Tek ELx800 microplate reader. Following correction for the blanks and background controls, optical density of test wells was used to express percentage proliferation relative to those left untreated. Experiments were repeated 3 times.

#### *2.2.5.3 Anchorage Independent Growth Assay (Colony Forming Assay)*

This assay uses a two gel system to study the ability of cells suspended in agar or methylcellulose to form colonies<sup>392</sup>, which can be extrapolated as a measure of malignant potential in vivo.

For the base gel, 1% (w/v) agar was microwaved and left in a water bath to cool to 37°C. An equal volume of solution containing 2x DMEM with 20% FCS was warmed to the same temperature. The solutions were mixed to give final concentrations of 0.5% (w/v) agar, 1x growth medium and 10% FCS. Appropriate amounts of supplemental reagents (eg iron and/or chelators) were added to achieve optimal stimulation concentrations (100mM FeSO<sub>4</sub> and 20mM haem), as determined by viability and proliferation assays (sections 4.2.2 and 4.2.3).

2ml of base agar per well was pipetted into 6 well plates, using 3 wells per experimental line. Care was taken to avoid air bubbles permeating the solution. Plates were allowed to cool and refrigerated at 4°C until adding the superficial agar (base agar can be kept refrigerated for up to 1 week).

The superficial gel solution was prepared as described above (substituting 0.7% (w/v) for 1% (w/v) agar, giving a final concentration of 0.35% (w/v)) and kept in a water bath at 37°C. Supplemental reagents were added as for the base gel.

Trypsinised cells were passed through a 50µm cell sieve (to prevent clumping) and counted, then sufficient added to the superficial gel solution to plate out at 1x10<sup>4</sup>/ml. Care was taken not to add the cells until the solution had cooled to 37°C. 0.5ml of superficial gel was gently placed onto the base gel.

Each well was photographed at 5 marked points at T=0 hours using a Zeiss Axiovert 40 CFL microscope and a camera with Axiovision Rel 4.6 imaging software. Plates were then incubated at 37°C for 2 weeks. Repeat photographs were then taken at the same points, colonies counted and their surface areas measured. Experiments were repeated 3 times.

#### *2.2.5.4 Cell Migration (Wound-Healing) Assay*

Cells were seeded into 6 well plates in 3ml/well of appropriate growth medium and allowed to form a confluent monolayer. At this point the medium was removed and a longitudinal wound

made in the monolayer using a white (0.1-10 $\mu$ l) pipette tip. The undersides of the wells were marked with a fine permanent marker to allow sequential photographs of the same point in the monolayer wound. 3 wells each were stimulated with 3ml of iron- or haem-loaded medium at concentrations dictated by previous proliferation and viability assays. A control was included. When necessary the media were changed every 24 hours until wound closure.

Photographs were taken at T=0 hours (immediately following wound formation and addition of stimulant media) with a Zeiss Axiovert 40 CFL microscope and a camera with Axiovision Rel 4.6 imaging software. Subsequent images were captured at time points determined by speed of migration of cells across the wound.

For each image the width of the wound was measured at 3 distinct points on the scale provided by the imaging software (N=9 points per experiment per test medium). The same 3 points were used for every image for robust comparison of migration rates under different conditions.

#### 2.2.5.5 Matrigel Invasion Assay

Matrigel is a reconstituted extract generated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma used as an analogue of true basement membrane in many cell phenotype assays<sup>393;394</sup>. It contains the constituents of basement membrane (collagens, laminin, proteoglycans) as well as growth factors and matrix-degrading enzymes (and their inhibitors)<sup>395;396</sup>.

Cells were incubated overnight in culture medium containing only 0.5% FCS (to eliminate any extrinsic enzymatic activity). Overnight incubation included iron- and haem-loaded media (at concentrations dictated by previous proliferation and viability assays), and a control. Commercial stock matrigel was thawed at 4°C and diluted 1:3 in cold serum-free cell culture medium. When handling the matrigel, pre-cooled pipette tips were used and plates kept on ice to prevent premature gel formation. Transwell inserts with 8.0 $\mu$ m pores were used, in keeping with previous invasion experiments performed on the same cell lines<sup>397-400</sup>.

The underside of the polycarbonate membrane in a transwell insert was covered with 15µl of diluted matrigel. This was allowed to set at 37°C for at least 40 minutes. Both sides of the membrane were then washed with PBS. Serum-starved cells were released into solution using cell dissociation buffer. They were centrifuged at 12 000rpm for 5 minutes to allow aspiration of the buffer then re-suspended in serum-free medium and counted using a Neubauer haemocytometer. The cell suspension was then diluted as necessary to give a concentration of  $0.5 \times 10^6$ /ml. 400µl of cell suspension was added to the insert, along with an appropriate amount of iron or haem. To the outside of the insert was added 400µl serum-free medium containing 5µg/ml fibronectin (to promote adhesion). Alamar Blue was added to the medium on both sides of the insert to a concentration of 10%. Alamar Blue is reduced by the products of cell metabolism to a form which can be made to fluoresce and can thus be used to quantify cell numbers<sup>401</sup>. The samples were left to invade at 37°C for 24-48 hours. Following invasion, 200µl of medium from outside the transwell was transferred to a 96-well plate. To quantify the total number of cells added, fluorescence in this sample was measured at wavelengths of 560nm for excitation and 590nm for emission using a Fluoroskan plate reader with Ascent software. Because liquid equilibrates between the two sides of the membrane, fluorescence inside and outside the transwell is the same and represents dye reduction due to the total number of cells present initially. Following migration, the transwell was moved to a fresh well containing PBS. A sterile cotton bud was used to wipe non-invaded cells from the inner surface of the membrane, which was then washed again in PBS. The transwell was placed in a well containing 800µl medium with 10% alamar blue. This medium contained 10% serum to ensure cells remained viable. The sample was left for a further 5 hours (or overnight) at 37°C before fluorescence was measured again to estimate the number of invaded cells. Relative fluorescence between total and invaded cells allowed calculation of percentage invasion.



## 2.2.6 Real-time PCR Analysis of mRNA Expression

### 2.2.6.1 RNA Extraction from Tissue Specimens and Cultured Cell Lines

**Tissue specimens:** were thawed and homogenized on ice with 1ml Trizol reagent using an UltraTurrax homogenizer.

**Cultured cells:** were grown in 6-well plates. After incubation culture media was aspirated and the cells washed 3 times with PBS. 500µl Trizol reagent was added and a cell scraper used to harvest the cells, which were thoroughly mixed with the Trizol and transferred into 1.5ml micro-centrifuge tubes.

Following addition of Trizol, fresh tissue specimens and cultured material were processed in the same way.

After 5 minutes' incubation, 200µl chloroform per 1ml Trizol was added and samples left at room temperature for a further 3 minutes. They were then centrifuged at 12000rpm for 15 minutes at 4°C and the colourless upper phase (containing total RNA) transferred to a fresh tube. The interphase (containing DNA) and lower red organic phase (containing protein) were transiently stored at 4°C for subsequent isolation of protein for Western blotting. 500µl isopropanol per 1 ml Trizol was added to the aqueous phase and the mixture left at room temperature for 10 minutes to precipitate RNA. The sample was centrifuged at 12000rpm for a further 10 minutes at 4°C and the supernatant discarded. The RNA pellet was washed in 1ml 75% (v/v) ethanol per 1ml Trizol and re-centrifuged at 7500rpm for 5 minutes at 4°C. The supernatant was again discarded and the pellet dried for 5 minutes at room temperature. The pellet was dissolved in an appropriate volume of nuclease-free water (depending on pellet size) and a Nanodrop ND1000 spectrophotometer was used to measure total RNA concentration and purity.

#### 2.2.6.2 cDNA Synthesis by Reverse Transcription

cDNA was synthesised using a Eurogentec reverse transcription kit according to manufacturer's instructions. An amount of solution containing 0.4µg total RNA was added to sufficient nuclease-free water to give a total volume of 8.1µl. This was added to 2µl reverse transcription buffer (10x), 4µl 25mM MgCl<sub>2</sub>, 4µl 2.5mM dNTP mix, 1µl random nonamer, 0.4µl RNase inhibitor and 0.5µl reverse transcriptase. Samples were loaded into a BioRad MyCycler thermocycling machine and cycled for 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C. 16µl nuclease-free water was added to the mixture, making a total of 36µl cDNA solution which was stored at -20°C until needed.

#### 2.2.6.3 TaqMan real-time qRT-PCR

TaqMan probes were available for all the genes of interest, using FAM (6-carboxyfluorescein) as a fluorophore and TAMRA (tetramethylrhodamine) as a quencher. See table 2-2 for probe and primer sequences.

A mastermix was prepared comprising 750µl sensimix (TaqMan DNA polymerase, dNTPs, MgCl<sub>2</sub>), varying volumes of probe (dictated by optimization reactions), 150µl each of forward and reverse primers, 7.5µl 18S probe, 15µl 18S forward/reverse primer mix and sufficient nuclease-free water to give a total volume of 1.4ml. Probes were supplied in 1.25pM solutions. Optimised volumes of probes are given in table 2-5, along with the corresponding quantity of water required for each mastermix.

1µl of each cDNA was pipetted in triplicate into an optical 96-well plate along with 14µl PCR mastermix. Plates were sealed with adhesive optical sealant film and centrifuged for a few seconds at 1200rpm to remove any bubbles from the bases of the wells. Plates were refrigerated or frozen if there was a delay in processing, but were re-centrifuged prior to

analysis. They were then taken to an Applied Biosystems 7500 FAST real-time PCR detection system and subjected to 40 cycles of PCR.

Following PCR, SDS software was used to generate Ct values for the gene of interest ( $C_{t_{\text{target}}}$ ) and 18S ( $C_{t_{\text{control}}}$ ) in each well. Relative quantification was then achieved as follows<sup>402</sup>:

A  $\delta C_t$  value was first obtained for every sample:

$$\delta C_t = C_{t_{\text{target}}} - C_{t_{\text{control}}}$$

To compare two samples (for example matched samples of normal and cancerous tissue), a  $\delta\delta C_t$  value was calculated:

$$\delta\delta C_t = \delta C_{t_{\text{sample 1}}} - \delta C_{t_{\text{sample 2}}}$$

*Note: the triplicate values for sample 1 in any given calculation were averaged to generate 3 values for  $\delta\delta C_t$ .*

As these values are generated from exponentially increasing quantities of DNA, relative amounts of original DNA (and hence sample mRNA) can thus be calculated:

$$\text{Relative mRNA expression} = 2^{-\delta\delta C_t}.$$

Gene	Probe Volume (μl)	Volume H <sub>2</sub> O (μl)
Dcytb	1.5	1.775
DMT1	1.2	2.075
Ferritin	1.5	1.775
Ferroportin	1.5	1.775
HCP1	1.5	1.775
Hepcidin	1.8	1.475
Hephaestin	1.2	2.075
IRP1	0.9	2.375
IRP2	0.9	2.375
LRP1	1.5	1.775
TfR1	1.2	2.075
BCRP	1.0	5.275
FLVCR	1.0	5.275

**Table 2-6 Optimized gene-specific probe volumes used in TaqMan PCR reactions**

All probes supplied as 1.25pM solutions. Required volumes of nuclease-free water also shown (to generate individual reaction volumes of 15μl). BCRP and FLVCR came as combined kits containing probe and forward/reverse primers and thus required larger volumes of diluent.

## **2.2.7 SDS-Polyacrylamide Gel Electrophoresis and Western Blotting**

### *2.2.7.1 Sample Preparation*

After extraction of the RNA-containing phase from homogenized samples, 300µl 100% ethanol per 1ml Trizol was added, mixed and left at room temperature for 3 minutes. After 5 minutes centrifugation at 2000rpm at 4°C the supernatant was moved to a fresh tube, leaving behind a DNA-containing pellet, which was discarded. 1.5ml isopropanol per 1ml Trizol was added to the supernatant, mixed and left for 10 minutes at room temperature. After centrifuging at 12000rpm for 10 minutes at 4°C, the supernatant was again discarded leaving a protein pellet. This was washed 3 times in 1ml 0.3M guanidine hydrochloride (made with 95% ethanol). The pellet was left for 20 minutes at room temperature in each wash, and re-centrifuged at 7500rpm for 5 minutes at 4°C before changing the wash solution. After the final wash the pellet was re-suspended in 100% ethanol, at which point it was frozen if not required for immediate use.

For analysis, the pellet was thawed and centrifuged at 7500rpm for 5 minutes at 4°C. The ethanol was removed by aspiration and the pellet dried under a vacuum for 5 minutes. It was then dissolved in 1% (w/v) sodium dodecyl sulphate and the solution centrifuged at 10000rpm for 10 minutes at 4°C to allow removal of an entirely liquid supernatant for Western blotting. This was subjected to a BCA protein assay (section 1.4.4.2) to standardise loading of protein samples. Before electrophoresis, samples were boiled for 5 minutes with 3x Laemmli sample buffer (0.0625M Tris HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue) at a ratio of 2:1 to denature the proteins.

#### *2.2.7.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis*

A Bio-Rad mini-PROTEAN tetra electrophoresis system was used. Resolving and stacking gels were prepared with compositions varying according to the molecular weight of the protein of interest (table 2-4). The resolving gel was poured into a 1.5mm casting tray and allowed to set. A layer of ethanol was poured onto the resolving gel to ensure a perfectly flat surface for receipt of the stacking gel. Once set, the ethanol was poured off and the stacking gel introduced along with a 10-well comb. Gels were submerged in running buffer (0.192M Glycine, 25mM Tris HCl, 0.01% (w/v) SDS, adjusted to pH 8.3) and 5µl of Amersham rainbow marker loaded into the first well of each. The remaining 9 wells were loaded with an amount of protein sample/laemmli buffer containing 10µg protein. Electrophoresis was commenced at 160V and continued until the dye front had reached the bottom of the gel.

#### *2.2.7.3 Western Blotting: Protein Transfer and Detection*

Following electrophoresis, gels were placed in a bath of transfer buffer (48mM Tris HCl, 20% (w/v) methanol, 39mM glycine, 0.0375% (w/v) SDS). Blotting pads and pre-cut filter paper were soaked in transfer buffer. Pre-cut PVDF membrane was labelled to identify and orientate the resulting protein transfers before being submerged in methanol for 30 seconds. It was passed through distilled and de-ionised water then immersed in transfer buffer. Transfer sandwiches were prepared with components kept soaked in transfer buffer at all times, and the sandwich was rolled before clamping to expel any air bubbles which would prevent transfer onto the membrane. Clamps were placed in the transfer apparatus and submerged in transfer buffer. Transfer was commenced at 100V for 60 minutes with ice packs in the tank to provide cooling.

Following transfer, membranes were blocked for 30 minutes with 5% (w/v) bovine serum albumin in Tris-buffered saline (made by adding 2.423g Trizma base and 8.006g NaCl to 80ml ultra-pure water, adjusting to pH 7.6 with HCl and topping up to 100ml with ultra-pure water) with Tween (TBST – 100ml x10 TBS, 900ml ultra-pure water and 1ml Tween). After blocking, membranes were incubated with optimised primary antibody solutions (in TBST) for one hour at room temperature, or overnight at 4°C. They were then washed 3 times in TBST for 10 minutes each before incubation with an appropriate secondary horseradish-conjugated antibody for 30 minutes at room temperature. A further 3 10 minute washes in TBST preceded 2 minutes bathing of the membrane in a 1:1 solution of ECL detection reagents 1 and 2. The membrane was then exposed to Amersham hyperfilm and developed using an X-OGRAPH X2 developer.

Densitometry was performed on the resulting immunoreactive bands using a BioRad GS800 densitometer and Quantity One software. Each sample was also processed for  $\beta$ -actin as a control, to ensure that initial protein quantification via the BCA assay had adequately normalised amounts of total protein. If necessary the entire process was repeated with adjusted volumes of protein in laemmli buffer being used for SDS-PAGE.

Gel Constituent	5% Stacking Gel	Separating Gel		
		8%	10%	12%
H <sub>2</sub> O (ml)	5.5	18.5	15.9	13.2
30% Acrylamide (ml)	1.3	10.7	13.3	16
1.5M Tris (pH8.8) (ml)	1	10	10	10
10% SDS (μl)	80	400	400	400
10% Ammonium Persulfate (μl)	80	400	400	400
TEMED (μl)	8	24	16	16

**Table 2-7 Composition of SDS-Polyacrylamide Gels**

Volumes of components given above are those required to make 40ml resolving gel and 8ml stacking gel; enough to make 4 SDS-polyacrylamide gels for electrophoresis.



### **2.2.8 Statistical Analysis**

Immunohistochemical analysis on archived material was performed on sections cut from 5 distinct blocks of normal breast tissue, 5 blocks of DCIS and 20 blocks of breast carcinoma. As the data generated were non-parametric, statistical analysis of differences in protein expression between the tissue types was performed using the Mann-Whitney U-test.

Protein and mRNA extracted from prospectively collected tissue was analysed in triplicate for every sample of each protein of interest on 3 separate occasions. Frequency histograms confirmed normal distribution of data generated from these experiments. Statistical significance was then tested using a 2-tailed Student's t-test.

All tissue culture experiments were performed using at least  $n=3$  samples in each experimental condition. Each experiment was repeated on 3 separate occasions to ensure reliability and reproducibility of results. Frequency histograms confirmed normal distribution of data generated from these experiments. Statistical significance was then tested using a 2-tailed Student's t-test.

Regardless of the statistical methods used, significance was accepted when the resulting p-value was 0.05 or less.

Microsoft Excel and SPSS v16 (IBM) software were used for statistical analysis.

## **CHAPTER 3: DYSREGULATION OF IRON AND HAEM TRANSPORT PROTEINS IN BREAST CANCER**

### **3.1 Introduction**

Iron was first implicated as a carcinogen when intra-muscular injections induced sarcomata in rats<sup>276</sup>, rabbits<sup>277</sup> and humans<sup>278</sup>. Epidemiological studies have related iron exposure to carcinogenesis in general<sup>279;280</sup> and to the development of specific epithelial tumours, including those of the colon<sup>311;403</sup>, kidney<sup>404;405</sup> and breast<sup>354;406</sup>. Many carcinomas exhibit over-expression of iron import proteins including TfR1, DMT1 and Dcytb and reduced expression of the export proteins ferroportin and hephaestin<sup>319;407;408</sup>.

Delineation of the iron transport apparatus in breast cancer specifically has so far revealed a number of important changes in expression of the proteins involved in transporting non-haem iron into and out of mammary epithelial cells. Ferritin levels are demonstrably up-regulated in breast cancer cells cultured in vitro relative to normal breast cell lines, implying higher levels of intra-cellular iron. Tissue culture and human tissue studies correlate a more aggressive phenotype and correspondingly poorer prognosis with higher expression of ferritin<sup>360;361;409</sup>.

The non-haem iron importers TfR1 and DMT1 are over-expressed in breast cancer relative to normal cells in tissue culture<sup>370;373</sup>, and this is coupled with a down-regulation of ferroportin expression, owing to over-expression of hepcidin<sup>366</sup>. The use of antisense oligonucleotides to suppress TfR1 mRNA expression has been shown to reduce intra-cellular iron concentration, and simultaneously inhibits both in vitro proliferation of cultured 4T1, MCF-7 and MDA-MB-231 breast cancer cells, and tumour growth and metastasis in the 4T1 murine mammary

adenocarcinoma model<sup>373;410</sup>. Similar results are seen with antisense oligonucleotides to suppress ferritin expression; these induce apoptosis in MCF-7 cells<sup>411</sup>.

The decrease in ferroportin expression in malignant breast cells seen in tissue culture is mirrored in vivo, where it has also been demonstrated to have value as a prognostic indicator (high levels of ferroportin, along with low hepcidin levels, place breast cancer patients in a favourable cohort)<sup>366</sup>. Transfecting breast cancer cells with ferroportin has been shown to significantly inhibit their growth when orthotopically implanted into the mouse mammary fat pad<sup>366</sup>.

The above evidence is gleaned from a combination of tissue culture experiments and analyses of tissue specimens. However, none of the papers quoted compared expression of the iron transport proteins in breast carcinoma samples with normal breast tissue harvested from the same patients. Observed changes could therefore reflect differences in basal expression of iron transport proteins between individuals rather than a modulation in expression in cancer compared to normal tissue.

Furthermore, there has been very little exploration of the role of haem importers (HCP1, LRP1) and exporters (BCRP, FLVCR) in breast cancer. LRP1 is thought to be involved in the development and invasion of metastases<sup>412-416</sup>, including those associated with breast cancer<sup>415;417</sup>. LRP1 gene polymorphism has been linked to increased susceptibility to breast carcinoma<sup>418</sup>, though to date the mechanism by which LRP1 functions in breast carcinogenesis and whether it is acting as a haem import protein are not known.

BCRP, a putative haem exporter, has been implicated in cancer, specifically those of the colon and cervix, where it has been shown to be down-regulated<sup>419</sup>. However, expression of BCRP in breast cancer and whether it functions as a haem exporter in this context has not yet been investigated. What evidence has been published demonstrates that BCRP can function as an

export protein and that this enables it to confer chemo-resistance on breast cancer cell lines<sup>265</sup>. However, it is also capable of extruding multiple other compounds from mammary cells<sup>420-423</sup>, including porphyrins<sup>272;275;424</sup>. No evidence has yet been published linking changes in expression of either HCP1 or FLVCR with any form of cancer.

### Chapter Aims

1. Determine the mRNA/protein expression profile of iron and haem import, export and storage proteins in prospectively collected matched specimens of normal breast tissue and cancer.
2. Determine the cellular location of these proteins in archived specimens of normal breast tissue (NB), ductal carcinoma in situ (DCIS) and breast cancer (BC).
3. Determine levels of intracellular iron in archived specimens of normal breast tissue, DCIS and cancer.

## **3.2 Expression Data**

### **3.2.1 Immunolocalisation of Iron and Haem Transport Proteins**

Paraffin-embedded sections of normal breast tissue, DCIS and breast carcinoma were subjected to immunohistochemistry.

Samples were obtained from 5 individual blocks of normal breast tissue, 5 blocks of DCIS and 20 blocks of breast carcinoma. 1 section from each block was evaluated for expression of every protein of interest. All 30 sections for each protein were processed concurrently using the same antibody solutions to ensure identical conditions

Optimised antibody concentrations are listed in table 2-1.

Localisation of immuno-staining within cells was compared between the three groups.

Semi-quantitative analysis allocated a score of 0 (no staining), 1 (weak), 2 (moderate) or 3 (strong) to each specimen.

Formal blinding was not undertaken as the samples arrived pre-labelled and the architectural differences between the 3 tissue types are profound and obvious even on cursory examination.

Staining intensity was quantified by the author and validated by a consultant histopathologist.

P values were calculated using the Mann-Whitney U-test.

### *3.2.1.1 Immunolocalisation of Iron Import Proteins*

#### Dcytb

Expression of Dcytb was both cytoplasmic and membranous, with stronger staining observed on the cell membranes in all three tissue types (figure 3.1). Semi-quantitative analysis suggested that expression of Dcytb was significantly increased in breast cancer relative to normal tissue with a positive mean fold change of 2.69 ( $p=0.004$ ). There was no difference between DCIS and normal tissue (table 3-1).

#### DMT1

Expression of DMT1 in normal breast tissue was largely cytoplasmic. Both DCIS and cancer specimens exhibited stronger staining on cell membranes than in the cytoplasm, although the avidity of staining in the cancer specimens made this difference negligible (figure 3.1). Cancer specimens also exhibited significant stromal staining, not seen in normal tissue. Semi-quantitative analysis showed DMT1 expression to be significantly increased relative to normal breast in the cancer sections with a mean fold change of 2.19 ( $p=0.015$ ). No other results were significant.

#### TfR1

There was little staining observed in the normal specimens. TfR1 was expressed in the cytoplasm of DCIS and carcinoma specimens. The cancer specimens also exhibited marked TfR1 expression on the cell membranes, as well as in the stroma, as for DMT1 (figure 3.1). Semi-quantitative analysis demonstrated significantly increased TfR1 expression in cancer specimens relative to normal tissue with a mean fold change of 2.16 ( $p=0.004$ ). Other differences were not significant.

Protein	Staining Intensity			Fold Change Relative to NB	
	NB	DCIS	BC	DCIS	BC
Dcytb	0.8+/-0.837	1.4+/-0.548	2.15+/-0.745	1.75	2.688*
DMT1	0.8+/-0.447	1.2+/-0.837	1.75+/-0.716	1.5	2.188*
TfR1	1.25+/-0.5	2.0+/-0.707	2.7+/-0.483	1.6	2.16*

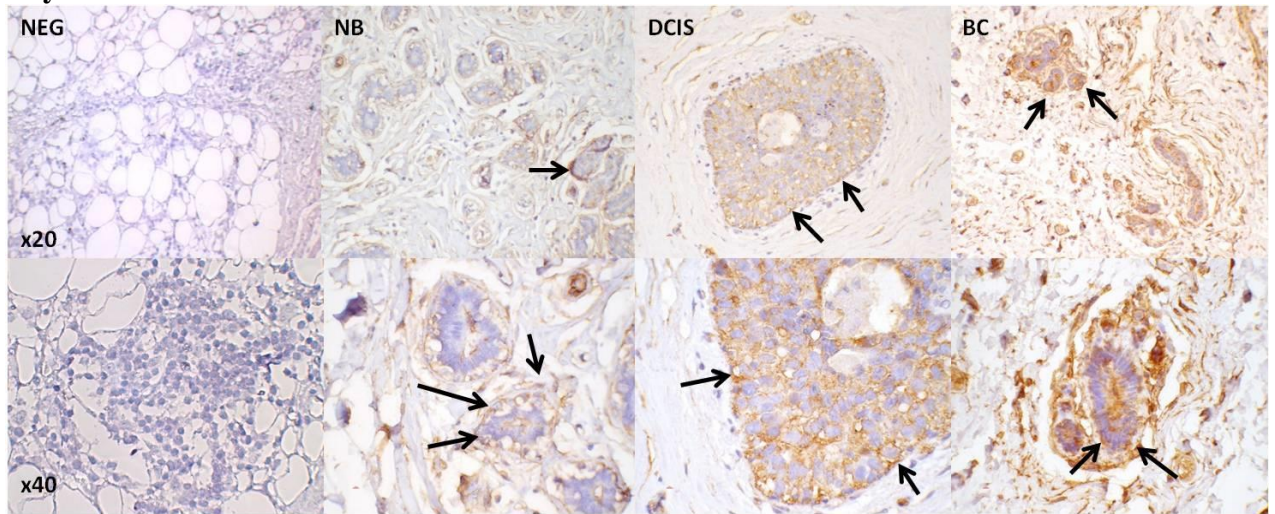
**Table 3-1 Semi-quantitative analysis of expression of the iron import proteins**

Intensity of immunohistochemical staining for the iron import proteins was compared between normal breast tissue (NB, n=5), DCIS (n=5) and breast carcinoma (BC, n=20). Results are expressed as a semi-quantitative score +/-2SEM (standard error of mean), and as a fold change relative to normal tissue.

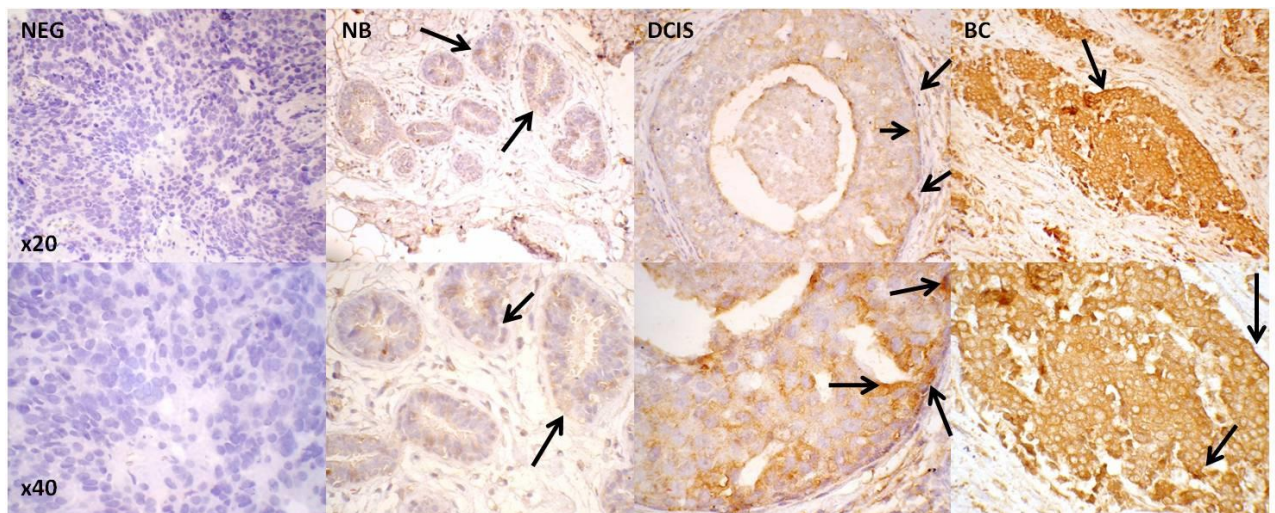
\* denotes statistical significance relative to normal breast (p<0.05).



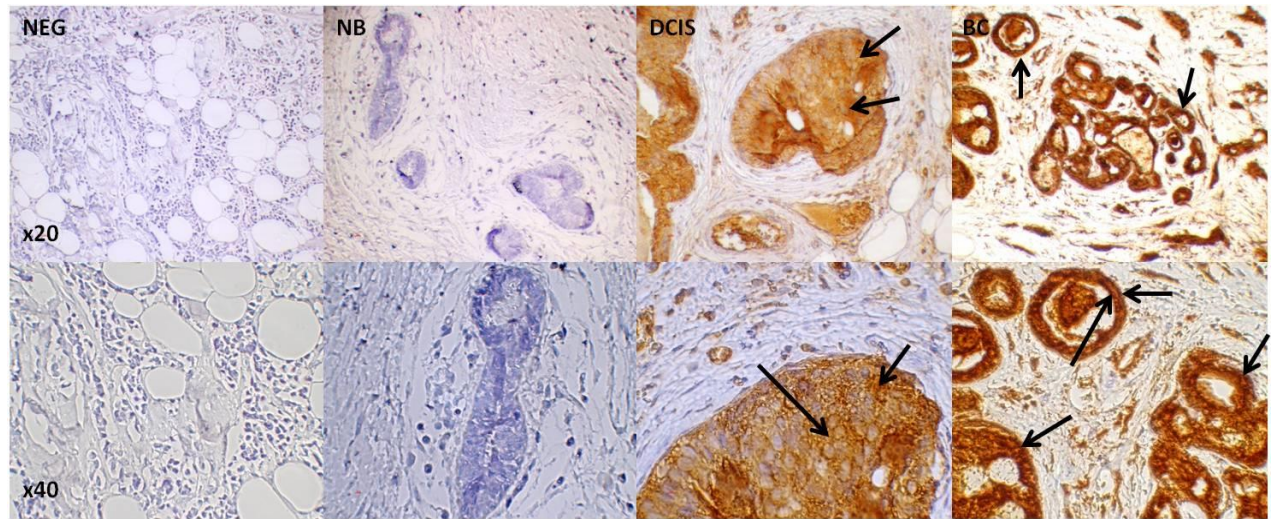
## Dcytb



## DMT1



### TfR1



**Figure 3.1 Immunolocalisation of the iron import proteins**

Immunohistochemistry was performed on paraffin-embedded sections to localise expression of Dcytb, DMT1 and TfR1 in normal breast (NB), DCIS and breast cancer (BC). Antibody concentrations for each are given in table 2-2. A negative control (NEG) was included by omitting primary antibody then processing the section in the usual manner with the relevant secondary antibody. Positive controls were mouse duodenum for Dcytb and DMT1 and mouse liver for TfR1. Images displayed are at x20 and x40 magnification.

### 3.2.1.2 Immunolocalisation of Haem Import Proteins

#### HCP1

Staining for HCP1 was cytoplasmic in all three specimen types. There was no significant difference in staining intensity between any of the three tissues.

#### LRP1

LRP1 expression was also cytoplasmic in normal breast, DCIS and carcinoma. There were no significant differences in staining intensity.

Protein	Staining Intensity			Fold Change Relative to NB	
	NB	DCIS	BC	DCIS	BC
HCP1	1.4+/-0.548	2.25+/-0.96	1.5+/-0.855	1.607	1.071
LRP1	0.8+/-0.837	1.4+/-0.548	1.3+/-0.675	1.75	1.625

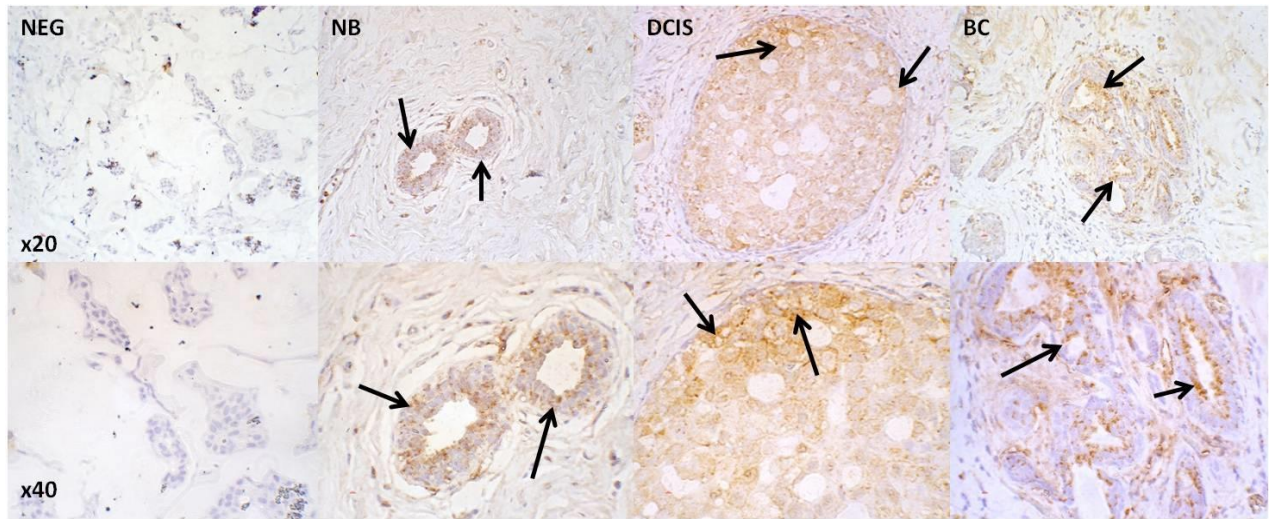
**Table 3-2 Semi-quantitative analysis of expression of the haem import proteins**

Intensity of immunohistochemical staining for the haem import proteins was compared between normal breast tissue (NB, n=5), DCIS (n=5) and breast carcinoma (BC, n=5). Results are expressed as a semi-quantitative score +/-2SEM (standard error of mean), and as a fold change relative to normal tissue.

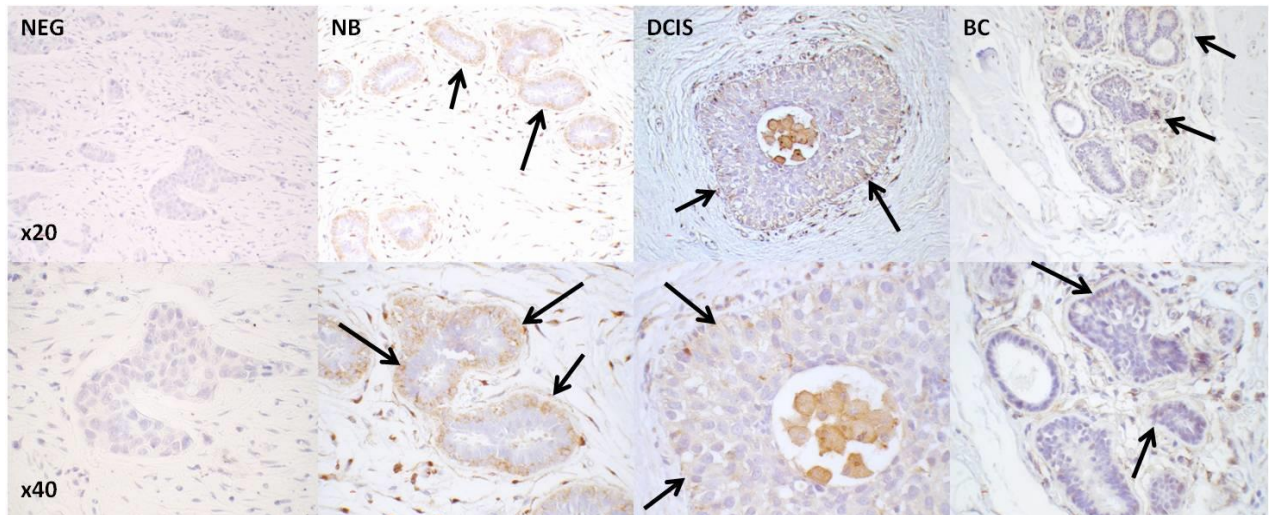
\* denotes statistical significance relative to normal breast (p<0.05).



### HCP1



### LRP1



**Figure 3.2 Immunolocalisation of haem import proteins**

Immunohistochemistry was performed on paraffin-embedded sections to localise expression of HCP1 and LRP1 in normal breast (NB), DCIS and breast cancer (BC). Antibody concentrations are given in table 2-2. A negative control (NEG) was included by omitting primary antibody then processing the section in the usual manner with the relevant secondary antibody. Positive controls were mouse stomach (HCP1) and liver (LRP1) Images displayed are at x20 and x40 magnification.

### *3.2.1.3 Immunolocalisation of Iron Export Proteins*

#### Ferroportin

All three tissue types demonstrated cytoplasmic and membranous staining for ferroportin, with membranous expression being more avid throughout. Semi-quantitative analysis did not reveal significant differences in expression between normal, DCIS or cancer specimens.

#### Hephaestin

Hephaestin expression was confined to the cytoplasm in all specimens. Expression levels analysed by semi-quantitative analysis were unchanged across the 3 tissue subtypes.

#### Hepcidin

Staining for hepcidin was cytoplasmic in all tissue types. No significant differences in expression were demonstrated by semi-quantitative analysis.

Protein	Staining Intensity			Fold Change Relative to NB	
	NB	DCIS	BC	DCIS	BC
Ferroportin	1.6+/-0.548	2.0+/-0	2.4+/-0.681	1.25	1.5
Hephaestin	1.8+/-0.837	2.4+/-0.548	2.45+/-0.609	1.33	1.36
Hepcidin	0.6+/-0.548	0.4+/-0.548	1.2+/-0.632	0.667	2.0

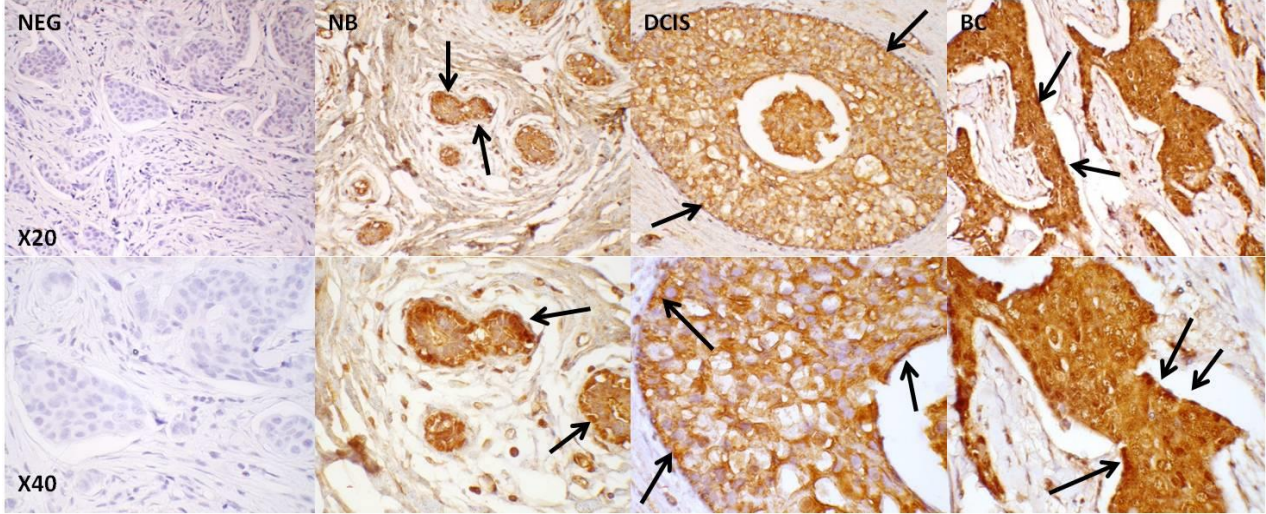
**Table 3-3 Semi-quantitative analysis of expression of the iron export proteins**

Intensity of immunohistochemical staining for the iron export proteins was compared between normal breast tissue (NB, n=5), DCIS (n=5) and breast carcinoma (BC, n=20). Results are expressed as a semi-quantitative score +/-2SEM (standard error of mean), and as a fold change relative to normal tissue.

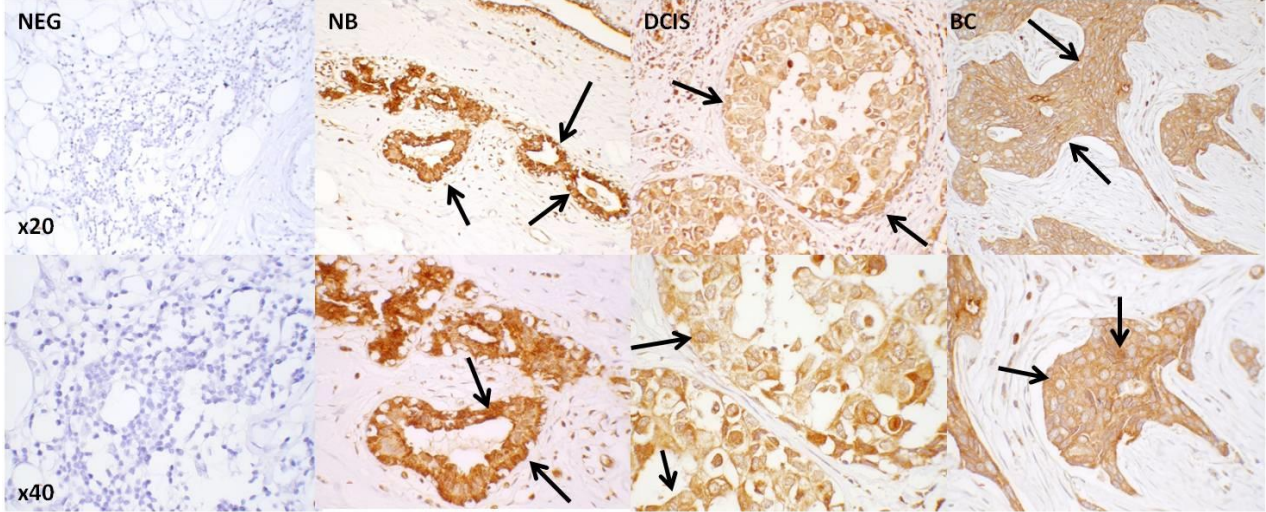
\* denotes statistical significance relative to normal breast (p<0.05).



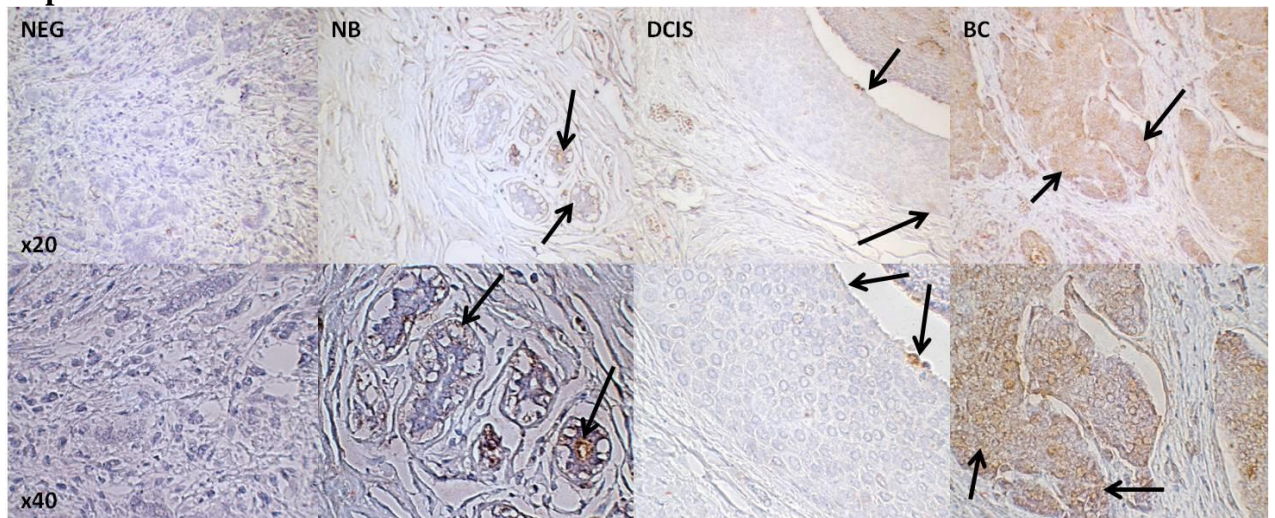
**Ferroportin**



**Hephaestin**



### Hepcidin



**Figure 3.3 Immunolocalisation of the iron export proteins**

Immunohistochemistry was performed on paraffin-embedded sections to localise expression of ferroportin, hephaestin and hepcidin in normal breast (NB), DCIS and breast cancer (BC). Antibody concentrations are given in table 2-2. A negative control (NEG) was included by omitting primary antibody then processing the section in the usual manner with the relevant secondary antibody. Positive controls were mouse liver (ferroportin and hepcidin) and colon (hephaestin). Images displayed are at x20 and x40 magnification.



#### 3.2.1.4 Immunolocalisation of the Haem Export Proteins

##### FLVCR

Staining for FLVCR was localised to the nuclei in all three tissue types. Semi-quantitative analysis did not reveal significant differences in expression.

##### BCRP

BCRP expression was cytoplasmic in normal breast tissue. However, in DCIS and breast cancer BCRP is localised at the cell border. Semi-quantitative analysis reported a statistically significant increase in expression of BCRP in breast cancer relative to normal tissue, with a mean fold change of 1.7 (p=0.026). There were no other significant differences.

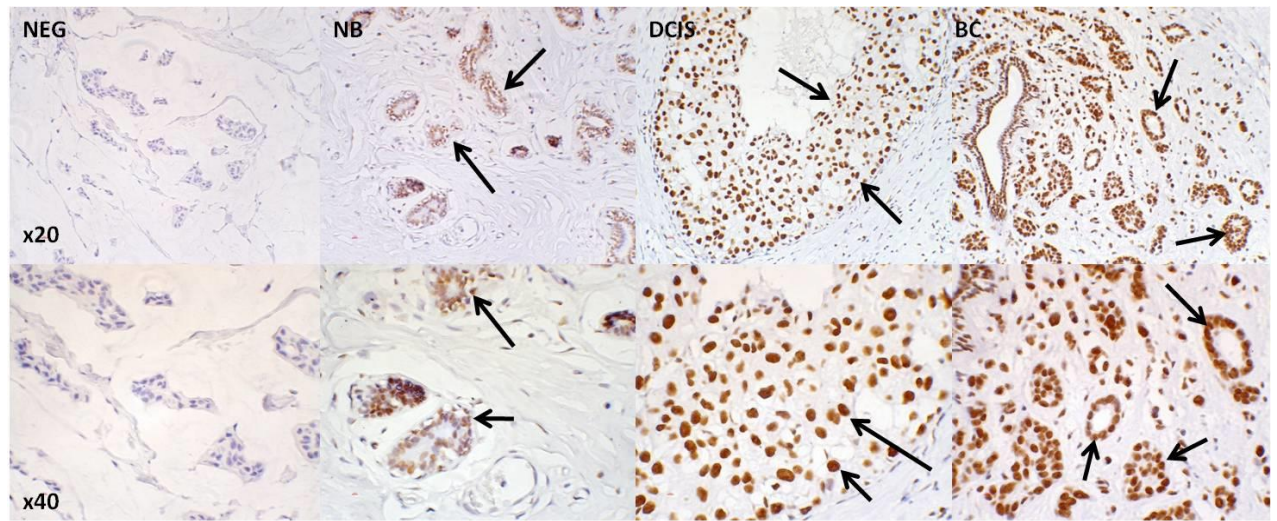
Protein	Staining Intensity			Fold Change Relative to NB	
	NB	DCIS	BC	DCIS	BC
FLVCR	2.0+/-0.816	2.2+/-0.447	2.3+/-1.054	1.1	1.15
BCRP	1.5+/-0.577	2.0+/-0	2.54+/-0.519	1.333	1.693*

**Table 3-4 Semi-quantitative analysis of expression of the haem export proteins**

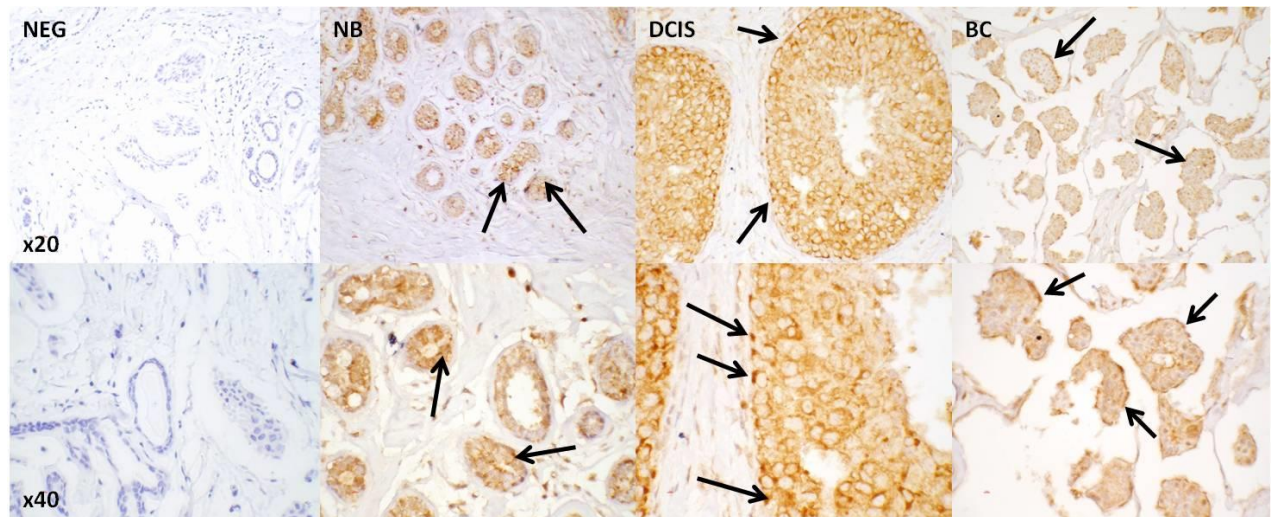
Intensity of immunohistochemical staining for the haem export proteins was compared between normal breast tissue (NB, n=5), DCIS (n=5) and breast carcinoma (BC, n=5). Results are expressed as a semi-quantitative score +/-2SEM (standard error of mean), and as a fold change relative to normal tissue.

\* denotes statistical significance relative to normal breast (p<0.05).

### FLVCR



### BCRP



**Figure 3.4 Immunolocalisation of haem export proteins**

Immunohistochemistry was performed on paraffin-embedded sections to localise expression of FLVCR and BCRP in normal breast (NB), DCIS and breast cancer (BC). Antibody concentrations are given in table 2-2. A negative control (NEG) was included by omitting primary antibody then processing the section in the usual manner with the relevant secondary antibody. Positive control for both was mouse kidney. Images displayed are at x20 and x40 magnification.

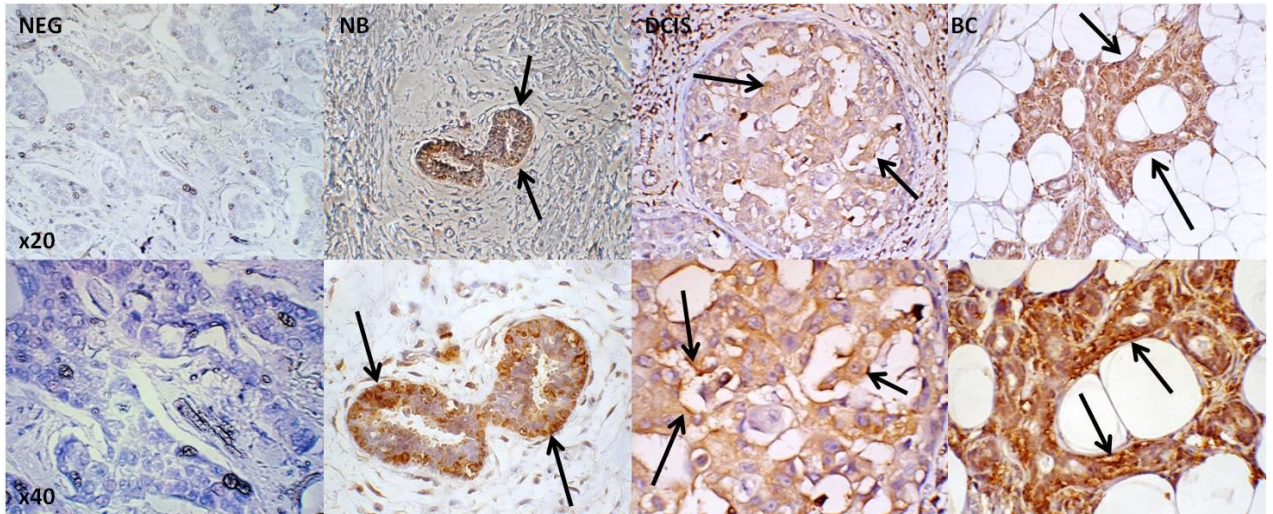
### 3.2.1.5 Immunolocalisation of Ferritin

Staining for ferritin in all three tissue types was cytoplasmic. No significant differences in expression between tissue types were revealed by semi-quantitative analysis.

Protein	Staining Intensity			Fold Change Relative to NB	
	NB	DCIS	BC	DCIS	BC
Ferritin	2.25+/-0.96	1.2+/-0.447	1.7+/-0.801	0.533	0.756

**Table 3-5 Semi-quantitative analysis of expression of ferritin**

Intensity of immunohistochemical staining for ferritin was compared between normal breast tissue (NB, n=5), DCIS (n=5) and breast carcinoma (BC, n=20). Results are expressed as a semi-quantitative score +/-2SEM (standard error of mean), and as a fold change relative to normal tissue. \* denotes statistical significance relative to normal breast (p<0.05).



**Figure 3.5 Immunolocalisation of ferritin**

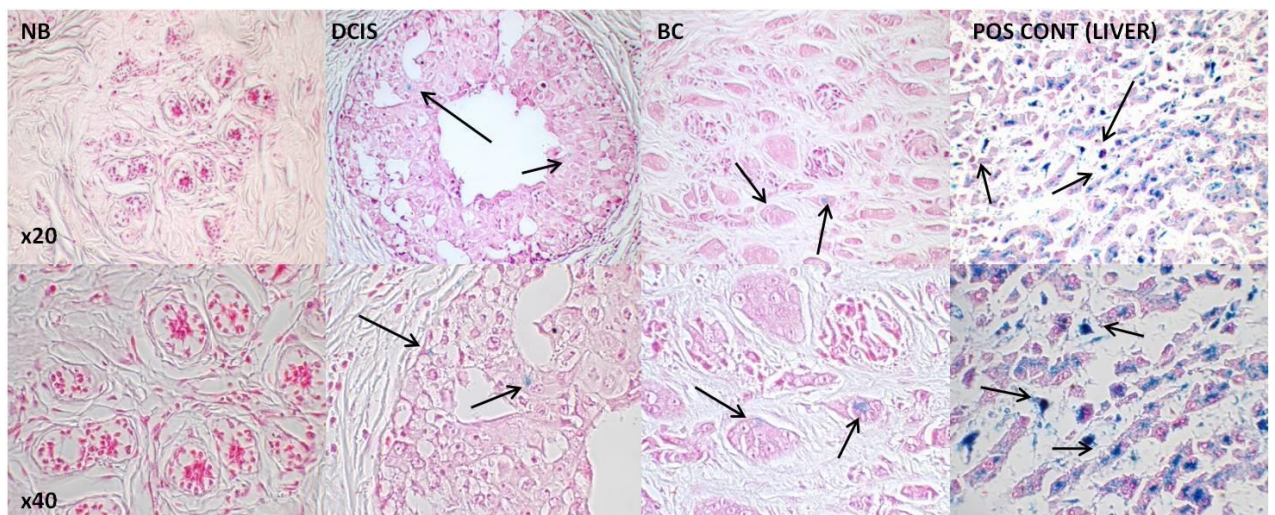
Immunohistochemistry was performed on paraffin-embedded sections to localise expression of ferritin in normal breast (NB), DCIS and breast cancer (BC). Antibody concentrations are given in table 2-2. A negative control (NEG) was included by omitting primary antibody then processing the section in the usual manner with the relevant secondary antibody. Positive control was mouse liver. Images displayed are at x20 and x40 magnification.



### 3.2.2 Intracellular Iron Content

Perl's staining was utilised to compare the intracellular iron content of normal breast tissue, DCIS and breast cancer. Normal liver was used as a positive control.

No staining for iron was observed in the normal breast sections. Although there were isolated deposits in the DCIS and cancer specimens, they were sparse, and of insufficient number to perform meaningful statistical analysis. This was the case throughout all the specimens analysed and was therefore unlikely to be due to tumour heterogeneity.



**Figure 3.6 Perl's staining for intracellular iron**

Perl's staining was performed to analyse the iron content of paraffin-embedded sections of normal breast (NB, n=5), DCIS (n=5) and breast cancer (BC, n=20). The arrows highlight areas of each section where iron has been liberated from ferritin and stained. Normal liver was used as a positive control (POS CONT) and demonstrates large amounts of intracellular iron. There are small pockets in the DCIS and cancer specimens, but no staining in normal breast. Images displayed are at x20 and x40 magnification.

### **3.2.3 Iron and Haem Transport Protein Expression in Matched Tissue Pairs**

SDS-PAGE and Western blotting were utilised to examine expression levels of the iron and haem import, export and storage proteins in breast carcinoma samples and matched normal tissue (see pages 58 and 73).

Owing to the finite amounts of protein generated from each collected tissue specimen, a number of the experiments did not utilise the entire library of samples.

All results are reported as mean expression in cancer relative to normal tissue (normal tissue results were standardised to 1 throughout).

$\beta$ -actin was used as a loading control.

Values are expressed as average relative expression  $\pm$  2SEM (Standard Error of Mean). P values were obtained using the student t-test.

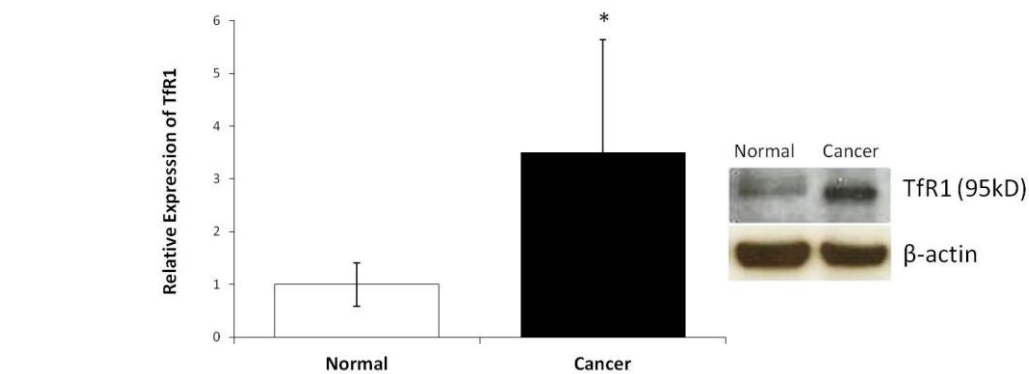
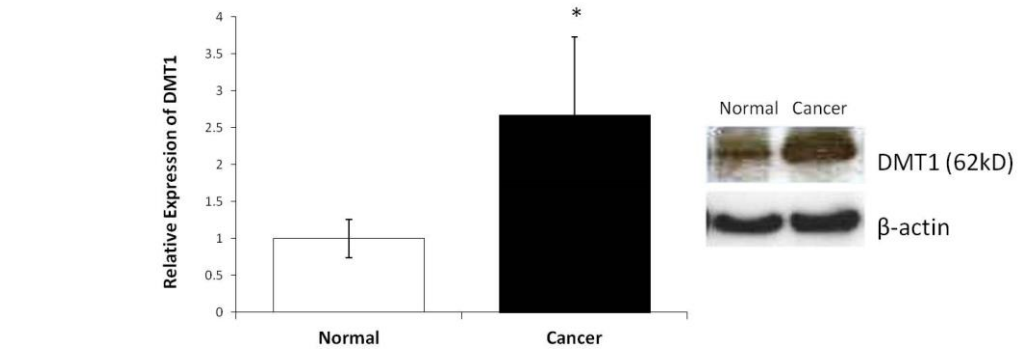
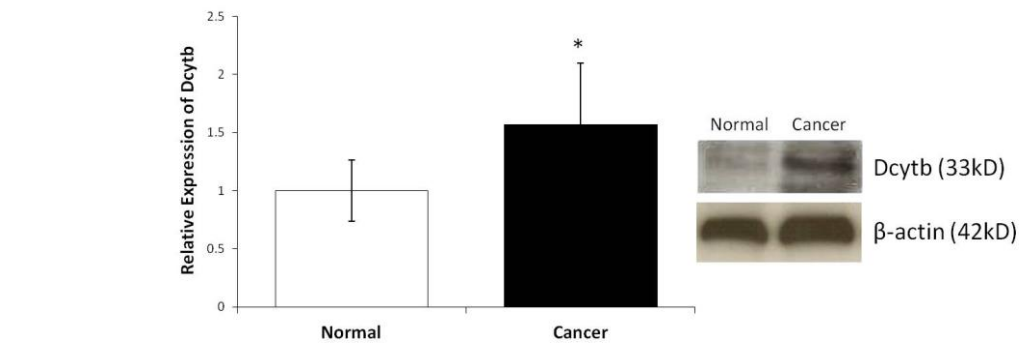
#### *3.2.3.1 Iron Import Proteins*

All three of the iron import proteins studied demonstrated significant over-expression in breast cancer compared to matched normal tissue (standardised to 1).

Dcytb expression in breast cancer specimens exhibited an increase of  $1.57 \pm 0.52$  ( $p=0.05$ ) relative to matched normal tissue.

There was a fold increase of  $2.67 \pm 1.06$  ( $p=0.008$ ) in expression of DMT1 in breast cancer compared to matched normal tissue.

TfR1 expression was also higher in cancer specimens relative to matched normal tissue with a mean fold change of  $3.51 \pm 2.12$  ( $p=0.036$ ).



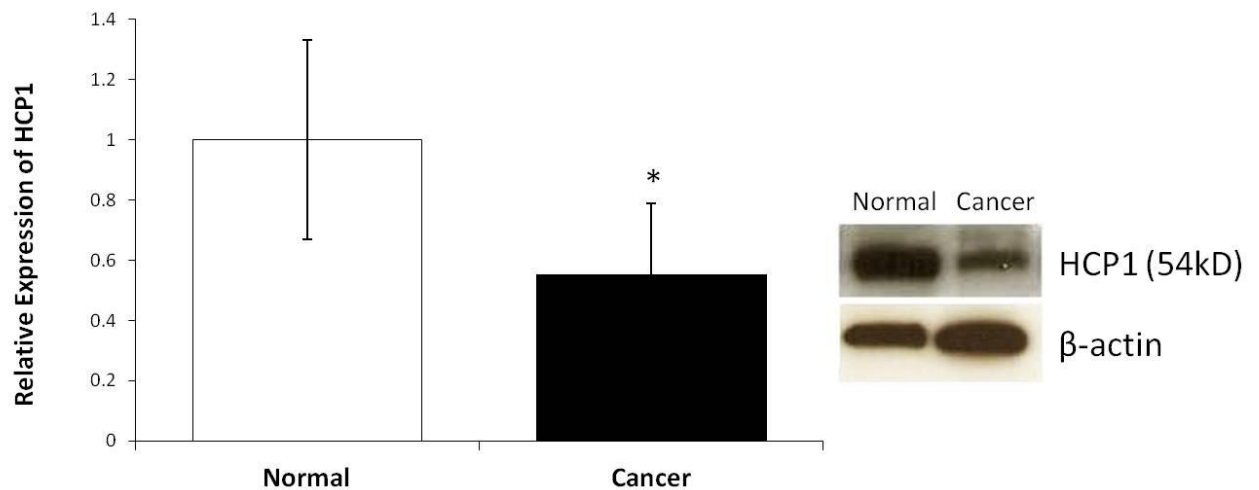
**Figure 3.7 Expression of iron import proteins in breast cancer relative to matched normal tissue**

Expression of Dcytb (**A**), DMT1 (**B**) and TfR1 (**C**) was significantly increased in breast cancer relative to matched normal breast tissue (n=13 matched samples).  $\beta$ -actin was utilised for normalisation purposes. \* denotes statistical significance ( $p < 0.05$ , student t-test).

### 3.2.3.2 Haem Import Proteins

Expression of HCP1 was down-regulated in cancer specimens with a 45% reduction relative to normal breast tissue (standardised to 1). Average relative expression was  $0.55 \pm 0.24$  ( $p=0.004$ ).

Despite multiple attempts on multiple sets of tissue lysates using antibodies from a number of sources, no adequate Western blots could be produced to investigate the relative expression of LRP1.



**Figure 3.8 Expression of HCP1 in breast cancer relative to matched normal tissue**

Expression of the haem import protein HCP1 is significantly down-regulated in breast cancer relative to matched normal tissue ( $n=10$  matched pairs).  $\beta$ -actin was utilised for normalisation purposes.

\* denotes statistical significance ( $p<0.05$ , student t-test).

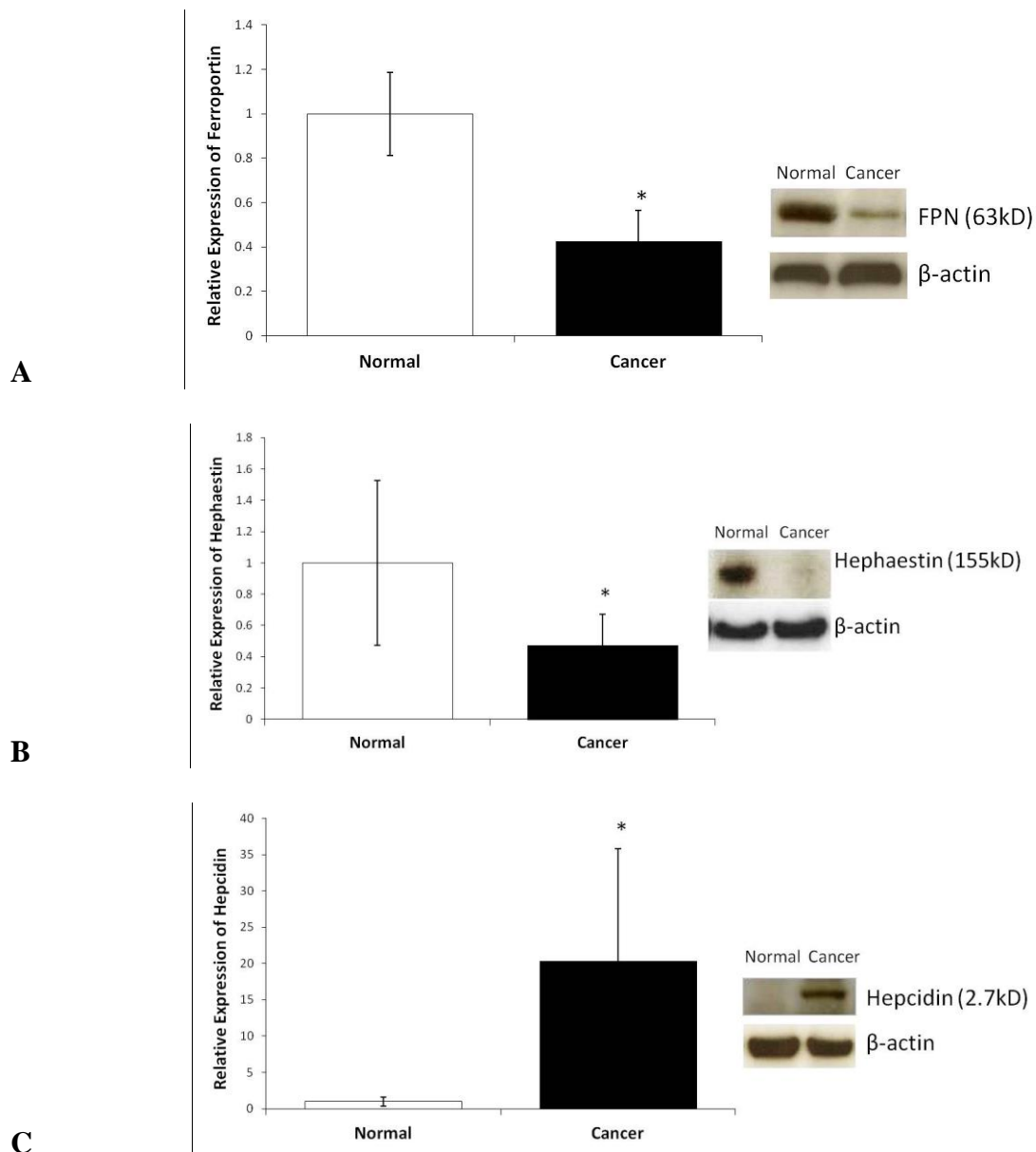


#### 3.2.3.3 Iron Export Proteins

Expression of the iron export protein ferroportin was reduced by 58% in breast cancer compared to matched normal breast tissue (standardised to 1). Average relative expression was  $0.42 \pm 0.14$  ( $p < 0.005$ ).

Expression of the ferroportin co-factor hephaestin was similarly inhibited, with an average relative expression of  $0.47 \pm 0.20$  ( $p = 0.006$ ), corresponding to a 53% reduction.

Conversely, expression of hepcidin was significantly increased in breast cancer relative to matched normal tissue. The average fold increase was  $20.33 \pm 15.43$  ( $p = 0.033$ ) compared to standardised normal tissue as throughout.



**Figure 3.9 Expression of iron export proteins in breast cancer relative to matched normal tissue**

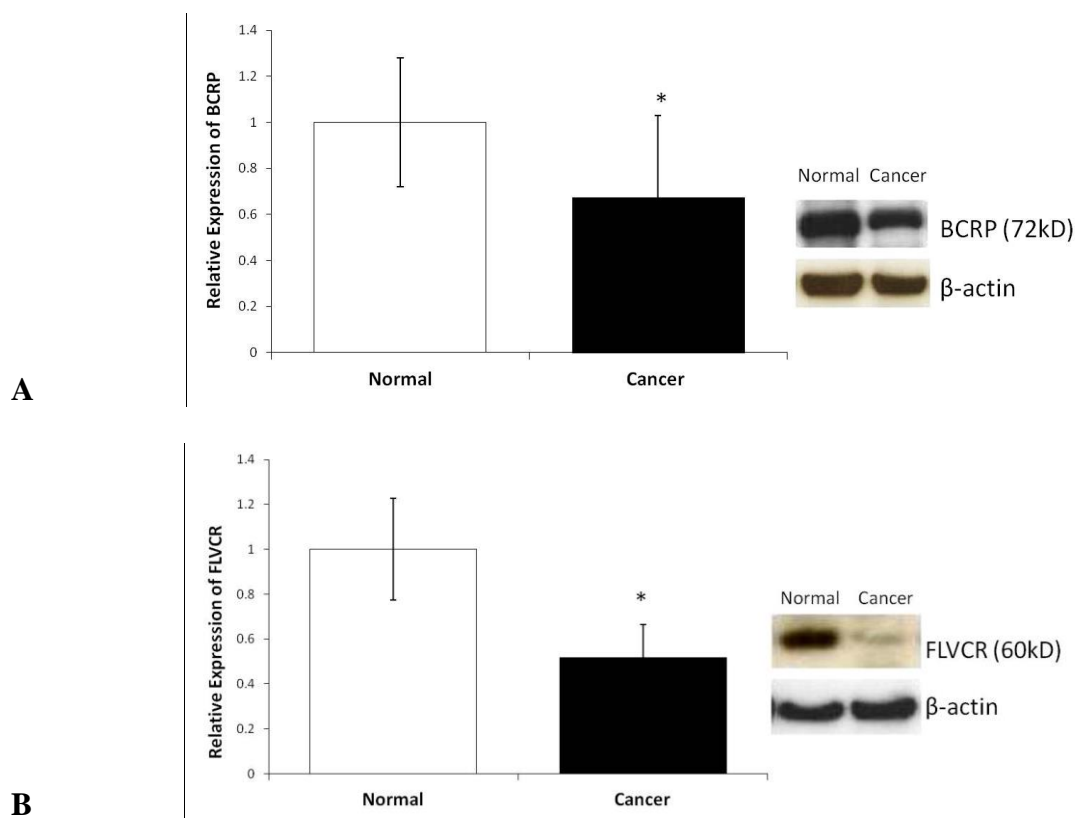
Expression levels of ferroportin (**A**, n=13 matched pairs) and hephaestin (**B**, n=5 matched pairs) were significantly repressed in breast cancer relative to matched normal tissue. Levels of hepcidin (**C**, n=10 matched pairs) were significantly increased.  $\beta$ -actin was utilised for normalisation purposes.

\* denotes statistical significance ( $p < 0.05$ , student t-test).

### 3.2.3.4 Haem Export Proteins

BCRP levels were significantly reduced (by 33%) in breast cancer compared to normal breast tissue. Average relative expression was  $0.67 \pm 0.35$  ( $p=0.005$ ) compared to normal breast standardised to 1.

Expression of FLVCR was also significantly down-regulated, with an average relative expression in breast cancer of  $0.52 \pm 0.15$  (a 48% reduction) compared with standardised, matched normal tissue ( $p<0.005$ ).

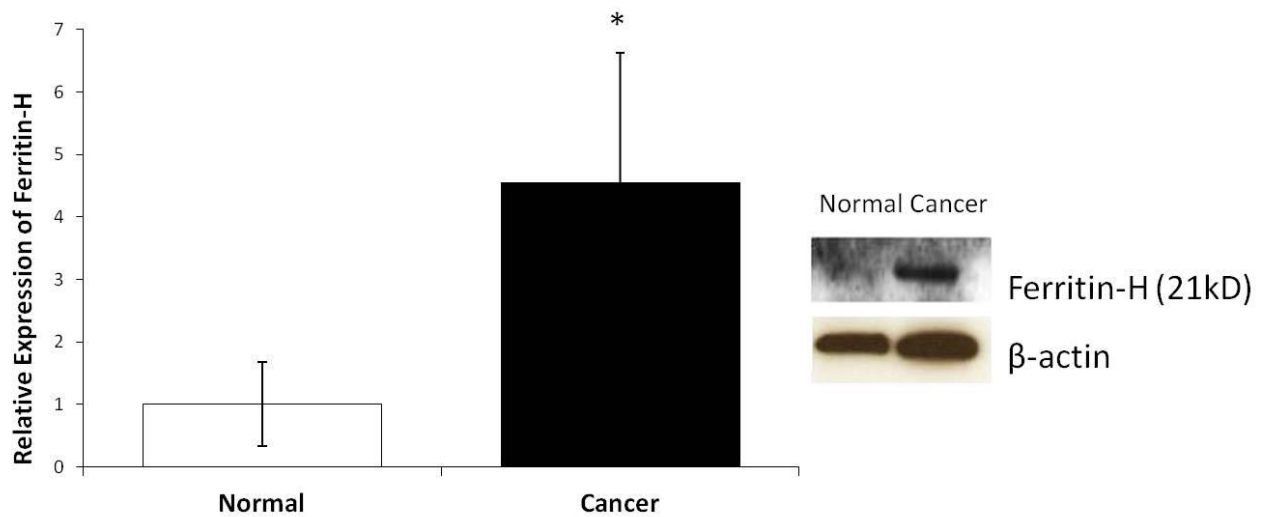


**Figure 3.10 Expression of haem export proteins in breast cancer relative to matched normal tissue**

Expression of BCRP (**A**,  $n=12$  matched pairs) and FLVCR (**B**,  $n=11$  matched pairs) was significantly down-regulated in breast cancer compared to matched normal breast tissue.  $\beta$ -actin was utilised for normalisation purposes. \* denotes statistical significance ( $p<0.05$ , student t-test).

### 3.2.3.5 Iron Storage

Ferritin-H was significantly over-expressed in breast cancer relative to matched normal tissue. There was a fold increase of  $4.55 \pm 2.07$  ( $p=0.012$ ) compared to normal samples standardised to 1.



**Figure 3.11 Expression of ferritin-H in breast cancer relative to matched normal tissue**

Ferritin-H is significantly over-expressed in breast cancer specimens relative to matched normal tissue ( $n=10$  matched pairs).  $\beta$ -actin was utilised for normalisation purposes.\* denotes statistical significance ( $p<0.05$ , student t-test).

### **3.2.4 Relative Expression of mRNA of Iron and Haem Transport Proteins**

Quantitative real-time polymerase chain reactions (qRT-PCR) were employed to elucidate changes in expression levels of mRNA coding for the various iron and haem transport proteins in the breast carcinoma samples and matched normal tissue (see pages 58 and 69).

Owing to the finite amounts of mRNA generated from each collected tissue specimen, a number of the experiments did not utilise the entire library of samples.

Results are expressed graphically as histograms demonstrating relative expression of mRNA in each matched pair, with summary histograms depicting the overall change in expression of mRNA for each protein.

Results are automatically normalised by the relative quantification PCR protocol such that expression in cancer specimens is always expressed relative to a value of 1 in normal breast tissue.

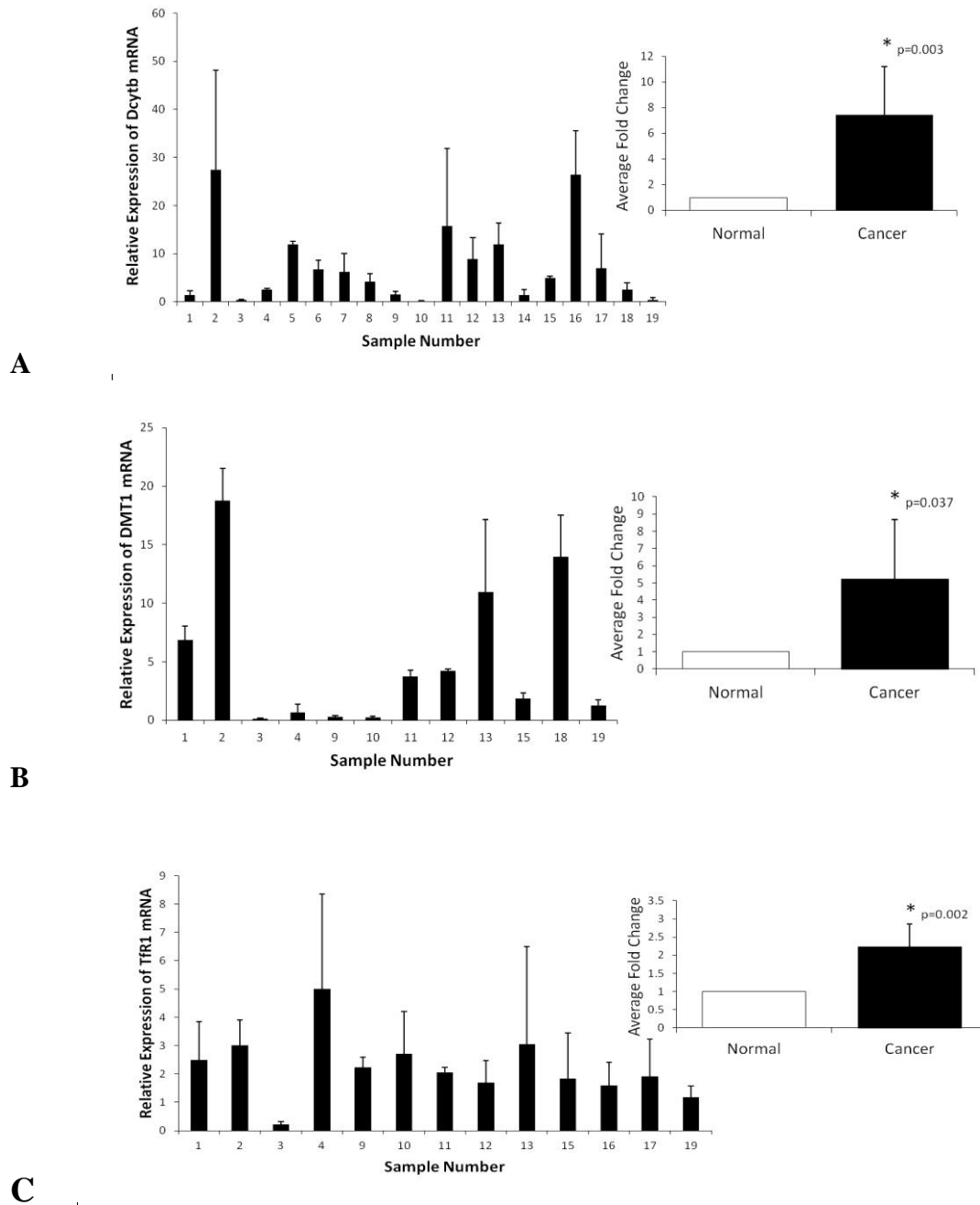
Fold changes are expressed as mean fold change $\pm$ 2SEM. P values were obtained using the student's t-test.

#### *3.2.4.1 Expression of mRNA for the Iron Import Proteins*

Dcytb mRNA expression was increased in 16 of 19 cancer specimens when compared to matched normal breast tissue (84%). The overall fold increase was  $7.45\pm 3.75$  ( $p=0.003$ ).

DMT1 mRNA was up-regulated in 8 of 12 (67%) breast cancer samples relative to matched normal tissue, equating to an average fold increase of  $5.25\pm 3.42$  ( $p=0.037$ ).

TfR1 mRNA demonstrated a fold increase of  $2.23\pm 0.63$  ( $p=0.002$ ) in expression, being over-expressed in 12 of 13 (92%) cancers relative to matched normal tissue.



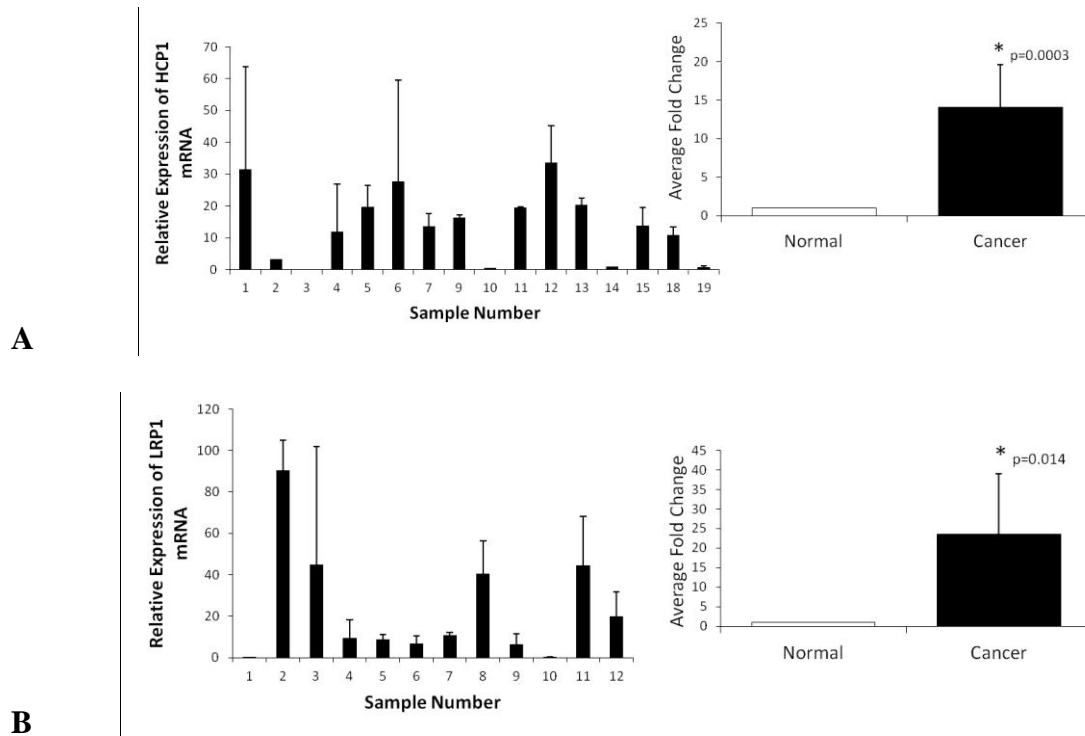
**Figure 3.12 Changes in expression of mRNA coding for the iron import proteins across matched tissue pairs**

Expression of mRNA coding for the iron import proteins Dcytb (**A**), DMT1 (**B**) and TfR1 (**C**) was significantly increased in breast cancer tissue relative to matched normal tissue. Histograms show relative expression  $\pm$  2SEM. \* denotes statistical significance ( $p < 0.05$ , student's t-test).

### 3.2.4.2 Expression of mRNA for the Haem Import Proteins

Expression of mRNA coding for HCP1 was up-regulated in 12 of 16 (75%) breast cancers relative to matched normal tissue, with an average fold increase of  $14.11 \pm 5.52$  ( $p=0.0003$ ).

There was increased expression of mRNA for LRP1 in 10 of 12 (83%) breast cancers compared to matched benign tissue; an average fold increase of  $23.60 \pm 15.46$  ( $p=0.014$ ).



**Figure 3.13 Changes in expression of mRNA coding for the haem import proteins across matched tissue pairs**

Expression of mRNA coding for HCP1 (**A**) and LRP1 (**B**) was significantly up-regulated in breast cancer specimens compared to matched normal tissue. Histograms show relative expression  $\pm$  2SEM. \* denotes statistical significance ( $p<0.05$ , student's t-test).

#### *3.2.4.3 Expression of mRNA for the Iron Export Proteins*

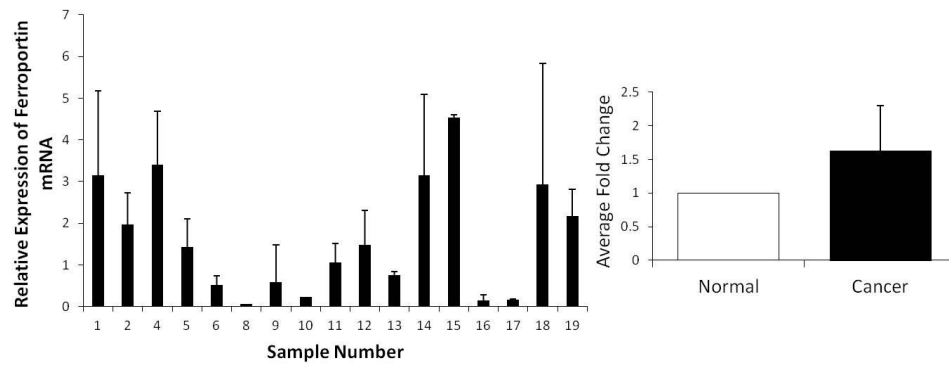
There was no significant change in expression of ferroportin mRNA in breast cancer relative to matched normal tissue.

Levels of mRNA coding for hephaestin were increased in the malignant samples in 14 of 19 (74%) matched tissue pairs. There was a statistically significant average fold increase of  $8.16 \pm 3.53$  ( $p=0.0007$ ).

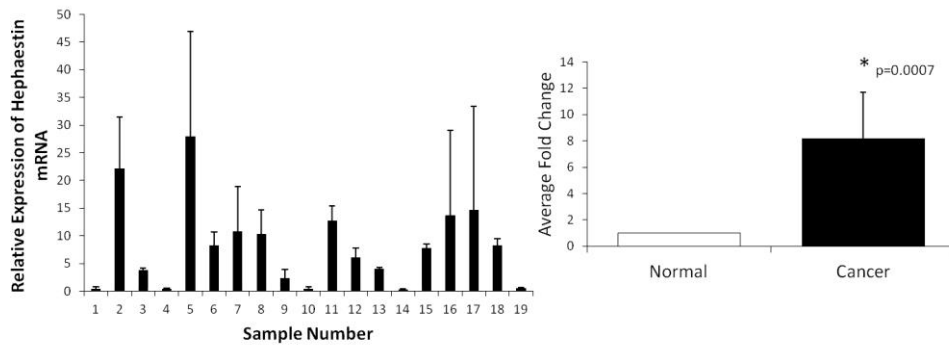
mRNA coding for the regulatory protein hepcidin was over-expressed in 8 of 11 (73%) malignant samples compared to matched normal tissue. There was a statistically significant average fold increase of  $14.48 \pm 8.74$  ( $p=0.012$ ).



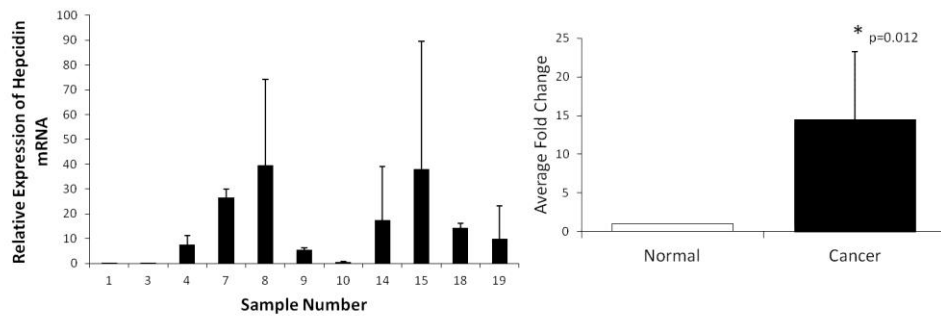
**A**



**B**



**C**



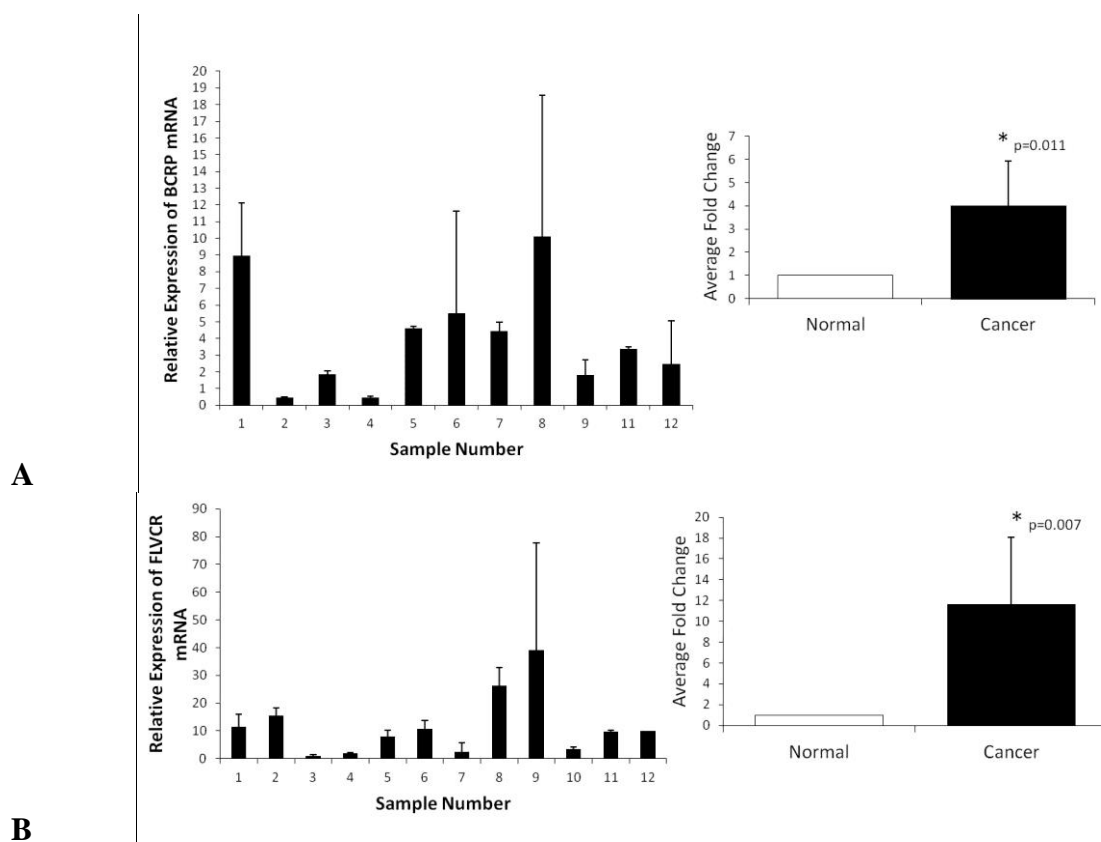
**Figure 3.14 Changes in expression of mRNA coding for the iron export proteins across matched tissue pairs**

Expression of mRNA coding for ferroportin (**A**) was not significantly changed in breast cancer relative to matched normal tissue. Expression of mRNA coding for hephaestin (**B**) and hepcidin (**C**) was significantly up-regulated in the malignant samples. Histograms show relative expression  $\pm$  2SEM. \* denotes statistical significance ( $p < 0.05$ , student's t-test).

#### 3.2.4.4 Expression of mRNA for the Haem Export Proteins

Expression of BCRP mRNA was elevated in 9 of 11 (82%) breast cancer specimens relative to their normal counterparts, with a statistically significant average fold increase of  $4.0 \pm 1.92$  ( $p=0.011$ ).

FLVCR mRNA was over-expressed in every one of 12 breast cancers relative to matched normal tissue, with a significant average fold increase of  $11.63 \pm 6.45$  ( $p=0.007$ ).

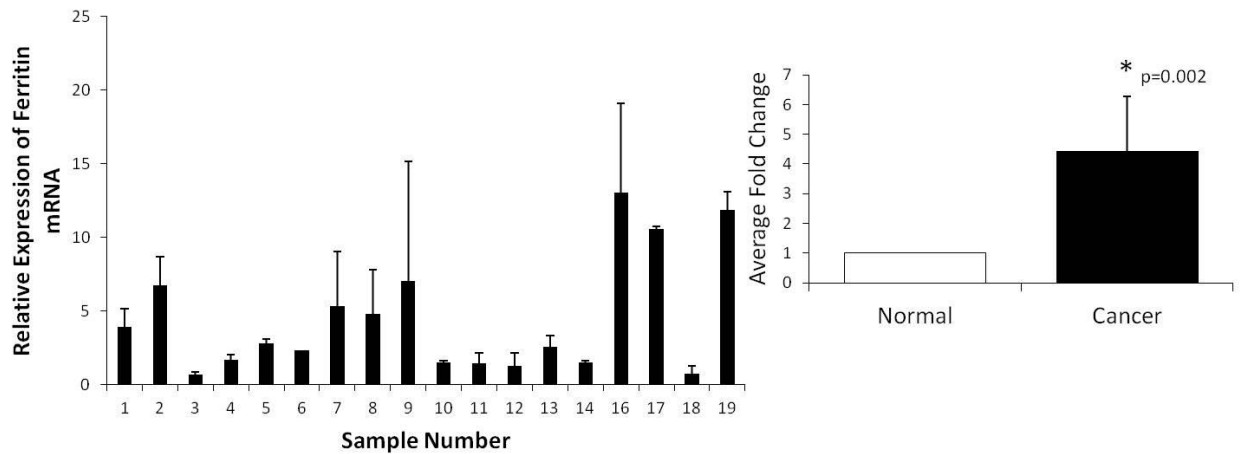


**Figure 3.15 Changes in expression of mRNA coding for the haem export proteins across matched tissue pairs**

Expression of mRNA for both BCRP (**A**) and FLVCR (**B**) was significantly up-regulated in breast cancer relative to matched normal tissue. Histograms show relative expression  $\pm$  2SEM. \* denotes statistical significance ( $p<0.05$ , student's t-test).

### 3.2.4.5 Expression of mRNA Coding for Ferritin

Levels of mRNA coding for ferritin were elevated in 16 of 18 (89%) breast cancers compared to matched benign tissue. There was a significant average fold increase in cancer of  $4.43 \pm 1.85$  ( $p=0.002$ ).



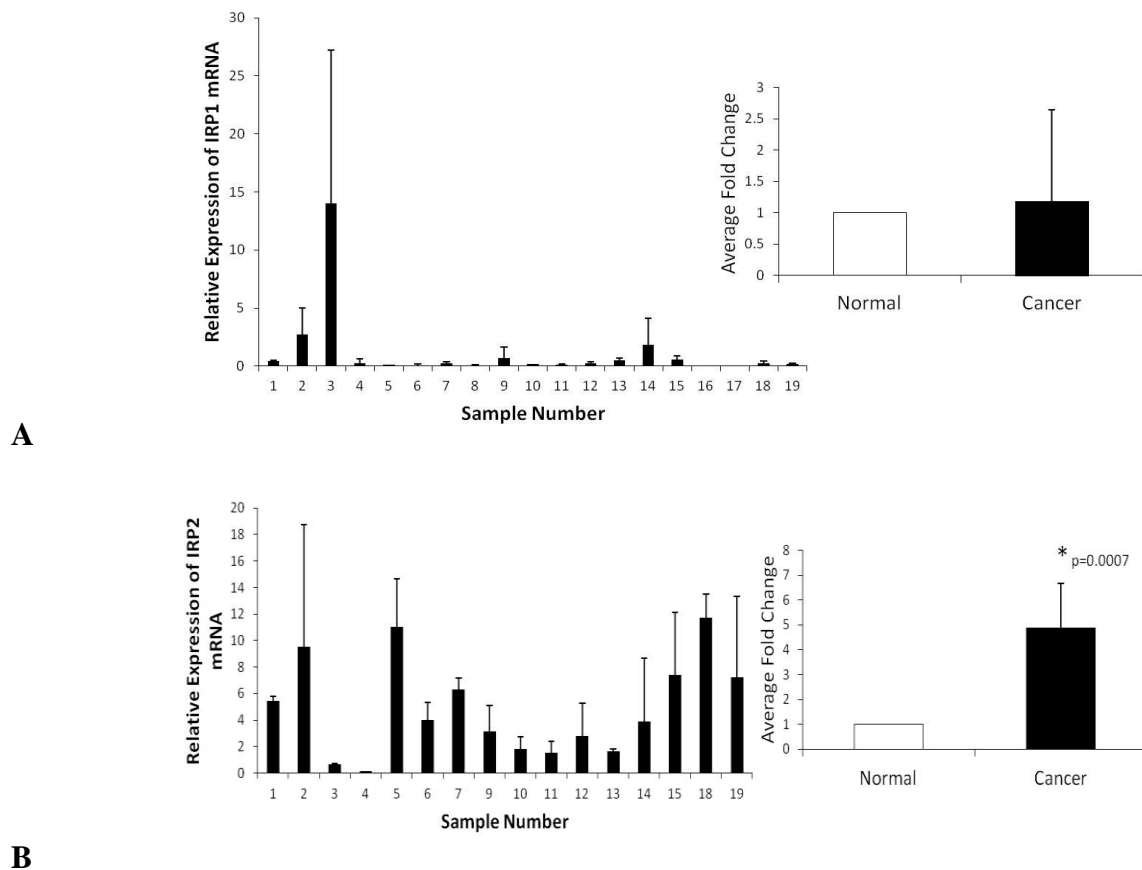
**Figure 3.16 Changes in expression of mRNA coding for H+L ferritin across matched tissue pairs**

There was a significant positive fold change in expression of the mRNA coding for ferritin in breast cancer compared to matched normal tissue. Results are displayed to show expression in the malignant specimen of each matched pair tested relative to its normal counterpart, and as an overall summary histogram. Histograms show average relative expression  $\pm$  2SEM. \* denotes statistical significance ( $p<0.05$ , student's t-test).

### 3.2.4.6 Expression of mRNA Coding for Iron Regulatory Proteins

Expression of IRP1 mRNA was unchanged across 19 matched tissue pairs with an expression in cancer relative to matched normal tissue of  $1.19 \pm 1.46$  ( $p=0.8$ ).

mRNA for IRP2 was elevated in 14 of 16 (88%) breast cancer samples relative to their matched normal tissue. There was a significant mean fold increase of  $4.89 \pm 1.77$  ( $p=0.0007$ ).



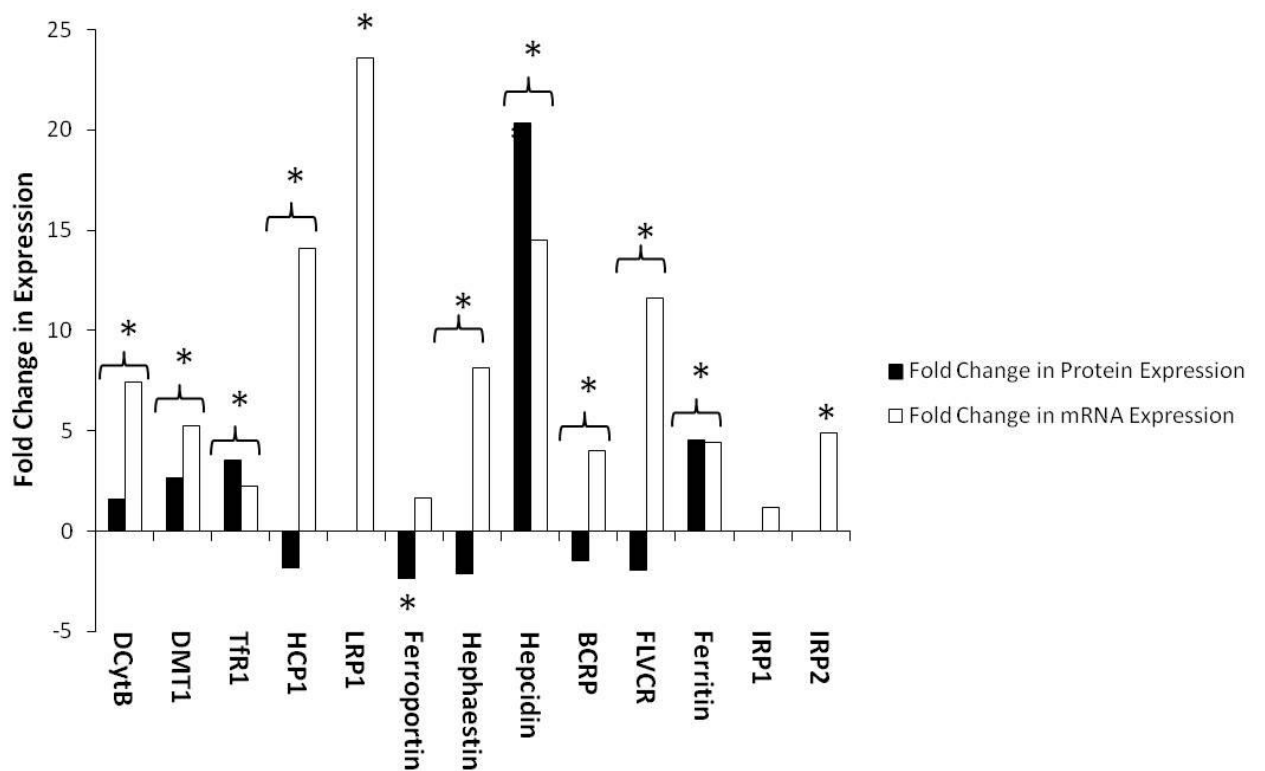
**Figure 3.17 Changes in expression of mRNA coding for the iron regulatory proteins across matched tissue pairs**

Expression of mRNA coding for IRP1 (**A**) was unchanged across matched tissue pairs. mRNA for IRP2 (**B**) was significantly over-expressed in breast cancer relative to normal tissue. Histograms show average relative expression  $\pm$  2SEM. \* denotes statistical significance ( $p<0.05$ , student's t-test).

	Expression of protein relative to matched normal tissue	Expression of mRNA relative to matched normal tissue
<b>Dcytb</b>	1.57 +/- 0.52 (p=0.05) *	7.45 +/- 3.75 (p=0.003) *
<b>DMT1</b>	2.67 +/- 1.06 (p=0.008) *	5.25 +/- 3.42 (p=0.037) *
<b>TfR1</b>	3.51 +/- 2.12 (p=0.036) *	2.23 +/- 0.63 (p=0.02) *
<b>HCP1</b>	0.55 +/- 0.26 (p=0.004) *	14.11 +/- 5.52 (p=0.0003) *
<b>LRP1</b>	N/A	23.6 +/- 15.46 (p=0.014) *
<b>Ferroportin</b>	0.42 +/- 0.14 (p<0.005) *	1.63 +/- 0.67 (p=0.078)
<b>Hephaestin</b>	0.47 +/- 0.2 (p=0.06) *	8.16 +/- 3.53 (p=0.0007) *
<b>Hepcidin</b>	20.33 +/- 15.43 (p=0.033) *	14.48 +/- 8.74 (p=0.012) *
<b>BCRP</b>	0.67 +/- 0.35 (p=0.005) *	4.0 +/- 1.92 (p=0.011) *
<b>FLVCR</b>	0.52 +/- 0.15 (p<0.005) *	11.63 +/- 6.45 (p=0.007) *
<b>Ferritin</b>	4.55 +/- 2.07 (p=0.012) *	4.43 +/- 1.85 (p=0.002) *
<b>IRP1</b>	N/A	1.19 +/- 1.46 (p=0.8)
<b>IRP2</b>	N/A	4.89 +/- 1.77 (p=0.0007) *

**Table 3-6 A summary of the changes in expression of the iron and haem import, export, storage and regulatory proteins and mRNA in breast cancer specimens relative to matched normal tissue**

Results are displayed as expression in cancer specimens relative to matched normal breast tissue (normalised to 1 in all cases) +/- 2SEM. \* denotes statistical significance (p<0.05, student t-test).



**Figure 3.18 Changes in expression of the iron and haem import, export, storage and regulatory proteins and mRNA in breast cancer relative to matched normal tissue**

Relative expression in cancer specimens is expressed as fold change relative to expression in normal breast tissue. \* denotes statistical significance ( $p < 0.05$ , student t-test).

	Relative Protein Expression	Relative mRNA Expression
<b>Dcytb</b>	↑	↑
<b>DMT1</b>	↑	↑
<b>TfR</b>	↑	↑
<b>HCP1</b>	↓	↑
<b>LRP1</b>	-	↑
<b>Ferroportin</b>	↓	→
<b>Hephaestin</b>	↓	↑
<b>Hepcidin</b>	↑	↑
<b>FLVCR</b>	↓	↑
<b>BCRP</b>	↓	↑
<b>Ferritin</b>	↑	↑
<b>IRP1</b>	-	→
<b>IRP2</b>	-	↑

**Table 3-7 Changes in expression of the iron and haem import, export, storage and regulatory proteins and mRNA in breast cancer relative to matched normal tissue**

The table summarises the significant changes in expression of the iron and haem transport proteins expressed numerically in table 3-6 and graphically in figure 3.18. The overall changes in protein expression would act to increase intracellular iron content. Some of these changes must be achieved by post-translational regulation given the differences in expression profile at the mRNA level.

### 3.3 Summary and Discussion

Mounting evidence suggests that dysregulation in the iron transport machinery is crucial to epithelial carcinogenesis. A number of studies demonstrate such a link in gastrointestinal carcinogenesis<sup>319;407</sup> and recent work has begun to demonstrate similar changes in breast cancer<sup>360;361;364;366;373;409-411</sup>.

Expression levels of Dcytb, DMT1 and TfR1 were significantly increased in breast cancer relative to normal breast tissue in our prospectively collected library of matched samples. These significant increases were also observed at the mRNA level. These results correlate with previous studies demonstrating increased expression of DMT1 and TfR1 in breast cancer, allowing increased transferrin receptor-mediated endocytosis of iron<sup>370;373</sup>. However, this is the first time expression levels have been compared in benign and malignant tissue obtained from the same individuals. Dcytb has not previously been studied in breast cancer and these results therefore represent a novel finding. Oesophageal and colorectal cancers have been shown to over-express Dcytb relative to normal tissue<sup>319;407</sup>, although as Dcytb is localised to the luminal cell membrane in both, this could be perceived as an upregulation of normal epithelial iron import machinery to cope with increased demand.

Immunohistochemistry on archived samples localised all 3 import proteins to the cell membranes in cancer, although both DMT1 and TfR1 also demonstrated marked immunoreactivity in the cytoplasm, likely reflecting accelerated turnover due to increased TfR-mediated endocytosis. Expression of Dcytb was also localised to the cell membrane in normal breast, although semi-quantitative analysis confirmed the significant increase in expression in cancer specimens revealed by Western blotting. DMT1 expression in normal breast tissue was largely cytoplasmic, indicating a translocation to the cell membrane in cancer. TfR1 expression in normal breast was negligible.



The observed changes reinforce the hypothesis that breast carcinoma cells have evolved a phenotype able to sequester increased amounts of iron from the circulation. In such a model, increased membranous TfR1 would allow increased influx of transferrin-bound iron through endocytosis. Increased cytoplasmic DMT1 would be required to process the excess endosomal iron resulting from increased import.

The increased expression of both Dcytb and DMT1 on the cell membranes in carcinoma may reflect a further step in evolution of normal mammary cells towards a phenotype geared to maximal iron uptake, attempting to mimic the gut luminal iron import mechanism to sequester unbound circulating iron.

A further interesting observation was the presence of significant staining for Dcytb, DMT1 and TfR1 in the stroma surrounding glandular tissue in breast cancer specimens particularly, although also to an extent in DCIS. This is not a feature that has previously been reported in the literature in relation to breast or any other cancer. It is conceivable that there is a peritumoural reaction in the connective tissue that induces such changes and this may be an area worthy of further study.

Immunolocalisation showed ferroportin to be located on the cell membrane in normal breast tissue as well as in cancer. Although there was no significant difference in levels of mRNA coding for ferroportin in cancer relative to matched normal tissue, Western blotting demonstrated a clear down-regulation in expression of the protein product, in keeping with results from previous studies<sup>366</sup>, and fitting the model of hepcidin-dependent internalisation and destruction of the protein product.

Similarly, Western blotting showed hephaestin expression to be significantly decreased in breast cancer tissue relative to matched normal samples, although paradoxically there appeared to be a significant increase in hephaestin mRNA. Given that the cells have adopted a

phenotype geared to increasing iron import, the mRNA up-regulation represents an appropriate response via the CDX2-regulated mechanism described on page 19. The decrease in protein levels therefore represents a post-translational moderation in hephaestin expression although the process is unclear. Immunohistochemistry revealed most of the staining to be cytoplasmic, in cancer specimens as well as normal tissue, which does not fit with its usual localisation to the cell membrane. This may well be a technical issue as previous groups have all demonstrated membranous expression in normal tissue, although expression in breast cancer has never been examined.

Immunohistochemistry revealed hepcidin expression to be localised to the cytoplasm in normal and malignant tissue. Previous studies have demonstrated cytoplasmic expression of hepcidin in the duodenum, although at a much reduced intensity than in hepatocytes, where synthesis occurs<sup>433</sup>. This duodenal staining is likely due to internalisation of the ferroportin-hepcidin complex which results in ferroportin degradation. A similar process must occur in both benign and malignant breast cells. Hepcidin expression was significantly increased in prospectively collected cancer specimens relative to matched normal tissue at both the protein and mRNA level, again reinforcing previously published data on breast cancer<sup>366</sup>, as well as replicating the situation previously described in colorectal carcinoma<sup>320</sup>.

The overall effect of the observed changes in expression of iron export proteins would be to decrease iron efflux from cells. Increased local expression of hepcidin would act to promote degradation of ferroportin. Thus, in the face of unchanged mRNA levels, amounts of the iron export protein will fall. Whether the discrepancies between expression of hephaestin and its mRNA are due to translational defects or increased degradation of the protein product, the consequence is a down-regulated iron export mechanism in the face of increased expression of iron import proteins.

Expression of the iron storage protein ferritin was significantly increased in breast cancer tissue relative to matched normal samples, replicating previously published results<sup>360;361;409</sup>. Immunohistochemistry demonstrated ferritin to be localised to the cytoplasm in all tissue types. There was a significant increase in mRNA expression. These changes are likely to be an appropriate response, regulated by the IRE/IRP interaction, to prevent increased intracellular iron levels (secondary to increased importer expression and decreased exporter function) causing damage via induction of ROS.

Immunohistochemistry showed both HCP1 and LRP1 to be localised to the cytoplasm of benign and cancerous breast tissue.

Western blotting on matched tissue pairs revealed HCP1 levels to be significantly reduced in cancer relative to normal tissue, although expression of HCP1 mRNA was significantly increased. Although HCP1 is recognised as a haem importer<sup>230</sup>, it is also known to transport a number of other compounds, including folate<sup>244</sup>, and it may be the case that the inhibition of HCP1 expression is a compensatory mechanism deployed in response to increased intracellular iron levels.

LRP1 mRNA levels were also significantly higher in breast cancer than matched normal tissue. Although LRP1 is known to have multiple functions, up-regulation in mRNA expression would permit breast cancer cells to import increased amounts of haem, thus contributing to the toxic intracellular milieu driving carcinogenesis. Unfortunately Western blotting with a number of antibodies raised against LRP1 failed to yield any results.

Immunohistochemistry showed expression of FLVCR to be nuclear in normal breast tissue, DCIS and breast cancer. Western blotting revealed a significant decrease in FLVCR expression in cancer relative to matched normal tissue, although levels of FLVCR mRNA were significantly up-regulated in malignant specimens compared to normal.

BCRP expression was shown to be largely membranous in breast cancer (and DCIS), whereas it was mainly cytoplasmic in normal tissue samples. Western blotting demonstrated significant down-regulation of BCRP expression in cancer relative to matched normal specimens, there was significant up-regulation in expression of BCRP mRNA.

Decreased expression of known haem exporter proteins would likely increase intracellular haem levels. Translocation of BCRP expression to the cell membrane in cancer specimens may suggest an attempt to export greater amounts of haem, although the decrease in overall quantity of the protein would be counter-productive and the varied role of BCRP in effluxing multiple compounds makes identification of its exact role in any context extremely challenging. The decreased expression of FLVCR in cancer combined with its nuclear location renders it ineffective as an exporter of haem from breast cancer cells. Nuclear localisation of FLVCR has not previously been reported and represents a novel finding, although the significance of this is uncertain. What can be stated is that confinement of FLVCR to the nucleus prevents it exporting haem from the cell and may contribute to an increase in intracellular haem. Although mRNA coding for both haem exporters is increased, post-translational regulation leading to decreased expression of the protein product would explain the observed results.

In this context, irrespective of the observed changes in expression of the putative haem import proteins, repression of the haem export proteins will lead to increasing intracellular haem which can be catabolised by HO-1 to liberate free iron. However, care must be taken in speculating on the degree to which these changes relate purely to haem metabolism given the varied functions carried out by these proteins<sup>264-266;270-272</sup>.

No antibody was available which yielded any positive immunohistochemistry or Western blotting results for the iron response proteins. However, an increase in IRP2 mRNA was

demonstrated in breast cancer samples relative to matched normal tissue. IRP1 mRNA expression was unchanged. Induction of IRP2 protein expression would cause cells to adopt an iron-deficient phenotype with up-regulation of TfR1 and DMT1, and inhibition of ferroportin expression, as seen in breast cancer<sup>366;367;369;370;373</sup>. The increase in expression of IRP2 mRNA suggests that the trigger for up-regulation of the protein product is inappropriate stimulation rather than failed degradation.

In summary, the changes observed in expression of iron and haem transport proteins combine to produce trafficking mechanisms for iron and haem which would act to increase intracellular concentrations of both. High levels of ferritin (disclosing an iron-replete intracellular environment) indicate that the increased expression of iron importers and down-regulation of exporters is inappropriate. This dysregulated expression profile appears to be multi-factorial with increased hepcidin down-regulating ferroportin on one hand and IRP2 stabilising TfR1 and DMT1 expression on the other.

Our data concerning non-haem iron transporters largely reinforce the results of previously published studies. As our comparisons of protein and mRNA expression between cancer and normal breast tissue are based on matched specimens taken from the same patient, all other variables are excluded and the differences observed must be due solely to altered expression profiles in malignant cells.

Haem transporters have not previously been studied in this context. However, down-regulation of haem exporter protein expression in the face of increased LRP1 expression would act to increase intracellular haem stores in a similar manner, although this may be partly offset by a down-regulation in HCP1 protein levels.

The likely consequence of these changes would be to increase the intracellular labile iron pool, driving carcinogenesis through the generation of reactive oxygen species, induction of oncogenic Wnt signalling and dysregulation of the cell cycle.

## **CHAPTER 4: CHANGES IN CELL PHENOTYPE INDUCED BY IRON AND HAEM LOADING IN VITRO**

### **4.1 Introduction**

The results reported in chapter 3 describe a modulation in expression of the iron and haem import and export proteins which could feasibly allow breast cancer cells to accumulate excess intracellular iron. These results support previously published studies in which breast cancer cell lines have been shown to up-regulate TfR1 and DMT1 relative to normal cells while down-regulating ferroportin<sup>373</sup>. It has been demonstrated that increased ability to sequester iron drives malignant mammary cells to behave more aggressively, reflected by the fact that higher levels of hepcidin, and subsequent down-regulation of ferroportin expression, bestow a poorer prognosis on breast cancer patients<sup>366</sup>. High levels of ferritin are also a poor prognostic marker<sup>361;409</sup>.

The observed modulation in expression of iron transport proteins would act to increase intracellular iron stores. A larger intracellular labile iron pool has the potential to mediate carcinogenesis through lipid peroxidation of membranes and DNA damage. In addition, increased intracellular iron levels are permissive for increased activity of ribonucleotide reductase, allowing higher rates of DNA synthesis and therefore cell cycling. De-regulation of ribonucleotide reductase expression has been shown to be an independent oncogenic stimulant in rodent studies<sup>425</sup>. The crucial role of iron in cell cycling has been described; in particular the incubation of malignant cells derived from a number of cancer subtypes (including breast) with an iron chelator reduced cellular expression of cyclins causing cell cycling to arrest<sup>296-298</sup>. Iron has also been shown to be a regulator of Wnt signalling; a major oncogenic signalling pathway implicated in a number of cancers including those of the breast<sup>323,359</sup>.

To date, very little work has been done to explore the changes induced in breast cell phenotype by increasing intracellular iron levels, or to investigate whether haem may play a part in modulating cell behaviour. The assumption has been that malignant cells manifest altered transport protein profiles due to their increased demand for iron. It has not been considered that iron and haem may drive a malignant cell phenotype, and that the altered expression profile of their transport proteins could be a causative factor in the development of malignancy. This could be mediated via direct toxicity, a permissive effect on ribonucleotide reductase function and cell cycling, or the induction of Wnt or some other signalling pathway. It has been shown that a proportion of breast cancer cells will arrest during cell cycling when incubated with chelators<sup>297</sup>. Iron chelators have also demonstrated an anti-proliferative action on cultured MCF-7 cells<sup>426</sup> and can potentiate the effects of traditional chemotherapy agents against breast cancer cell lines in vitro<sup>379</sup>. However, the effect of iron chelation on many of the phenotypic traits associated with cancer cells has yet to be investigated. The behaviour of breast cancer cells in terms of their proteomic response to increasing iron levels also remains to be formally reported.



## Chapter Aims

1. Determine whether benign and malignant mammary cell lines can import iron and haem and whether the efficacy of iron import differs between the two.
2. Investigate the effects of increased intracellular iron on the following aspects of cell phenotype (in benign and malignant lines):
  - a. Viability
  - b. Proliferation
  - c. Anchorage independent growth
  - d. Migration
  - e. Invasion
3. Determine whether any observed changes in cell phenotype are affected by iron chelation with deferasirox (ICL670A).
4. Examine the effects of iron, haem and iron chelators on cellular expression of the iron and haem transport proteins.

## **4.2 The Effect of Iron, Haem and Iron Chelation on Cell Phenotype**

### **4.2.1 The Effects of Iron and Haem Loading on Intracellular Iron Concentration**

To determine whether mammary cells can take up extracellular iron, cells were incubated with iron and haem at varying concentrations and time intervals.

We utilised HB2, MCF-7 and MDA-MB-231 cells in this and all other tissue culture experiments. Benign HB2 cells are derived from normal breast epithelium. MCF-7 cells are of intermediate malignant phenotype and are derived from invasive ductal carcinoma. MDA-MB-231 cells are also derived from invasive ductal carcinoma but exhibit a more malignant phenotype.

Each line was incubated with haem at 20, 40 and 80 $\mu$ M and FeSO<sub>4</sub> at 50, 100, 150 and 200 $\mu$ M for 24, 48 and 72 hours. 6 well plates were used, 3 wells for each experimental condition.

Experiments were repeated 3 times.

Ferrozine and BCA protein assays were performed on recovered cell pellets (see sections 2.2.4.1 and 2.2.4.2), allowing iron content to be expressed as nmol Fe per unit protein and compared to control conditions.

## HB2

Incubation with FeSO<sub>4</sub> increased intracellular iron content at all concentrations and time periods ( $p<0.005$ ). There were no differences in iron content following incubation with haem at any of the concentrations or time periods studied.

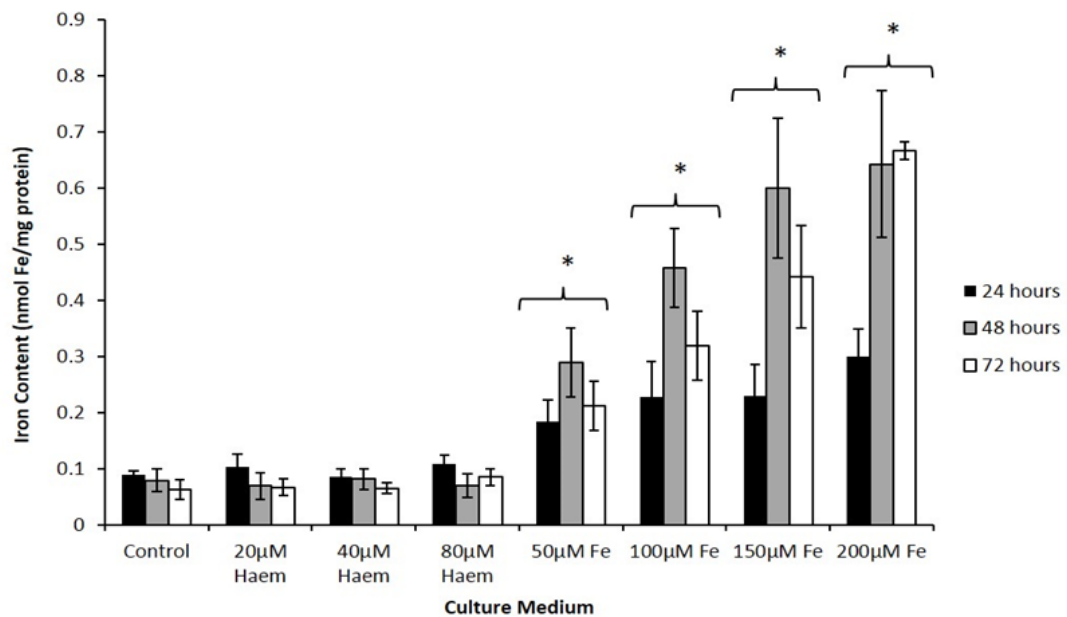
## MCF7

Incubation with FeSO<sub>4</sub> at all concentrations and time periods increased intra-cellular iron content ( $p<0.05$ ). Culturing with 80 $\mu$ M haem increased intra-cellular iron at all time points ( $p<0.05$ ). 40 $\mu$ M haem increased cellular iron levels at 24 ( $p=0.01$ ) and 48 ( $p=0.004$ ) hours while 20 $\mu$ M haem was only effective at 48 hours ( $p=0.036$ ).

## MDA-MB-231

Incubation with FeSO<sub>4</sub> increased cellular iron content over all concentrations and time periods except in cells incubated with 50 $\mu$ M FeSO<sub>4</sub> for 72 hours ( $p<0.05$ ). 40-80 $\mu$ M haem increased cellular iron levels at all time points ( $p<0.05$ ). 20 $\mu$ M haem only increased cellular iron levels at 24 hours.

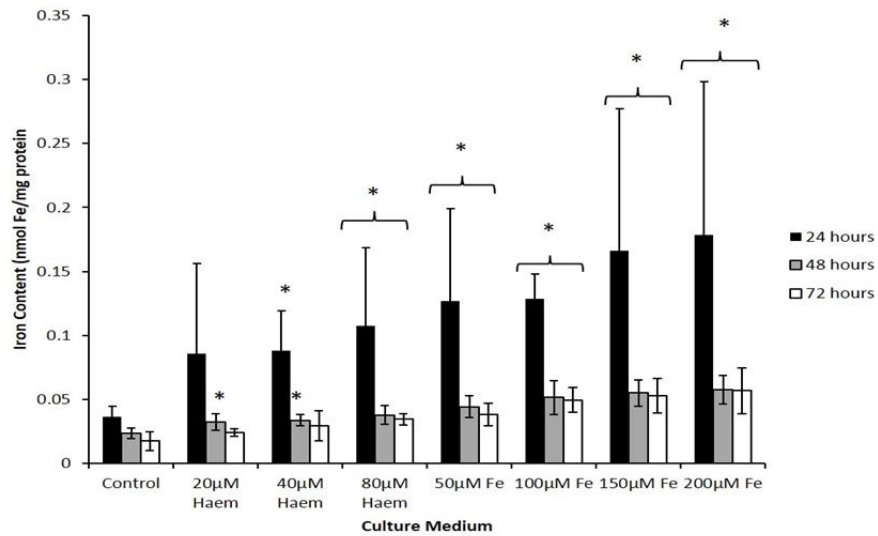
## HB2



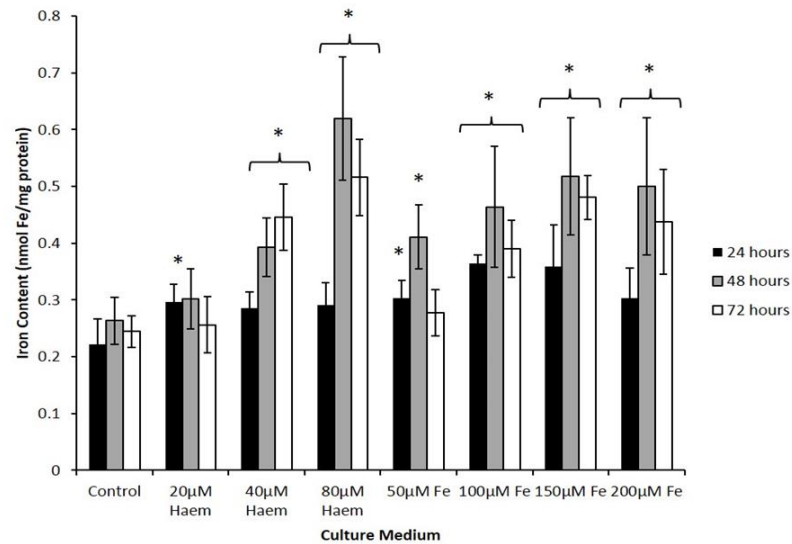
**Figure 4.1 Changes in intracellular iron content of benign breast cells induced by iron and haem loading**

Benign HB2 cells showed no increase in intra-cellular iron content in response to incubation with any concentration of haem for any period of time. Incubation with FeSO<sub>4</sub> significantly increased cellular iron levels in every experimental arm. Statistical significance ( $p < 0.05$ ) is denoted by \* (student t-test). Results are expressed graphically as mean intra-cellular iron content  $\pm$  2SEM.

## MCF7



## MDA-MB-231



**Figure 4.2 Changes in intracellular iron content of malignant breast cells induced by iron and haem loading**

MCF7 and MDA-MB-231 cells were capable of importing both iron and haem to increase their intracellular iron content at a variety of concentrations and time intervals. Statistical significance ( $p < 0.05$ ) is denoted by \* (student t-test). Results are expressed graphically as mean intra-cellular iron content  $\pm$  2SEM.

#### **4.2.2 The Effects of Iron and Haem Loading on Cell Viability**

An MTT assay was utilised to explore the effects of iron and haem loading on the viability of benign and malignant cells as described in section 2.2.5.1. Benign HB2 and malignant MCF7 and MDA-MB-231 cells were incubated with haem at 20, 40 and 80 $\mu$ M and FeSO<sub>4</sub> at 50, 100, 150 and 200 $\mu$ M for 24, 48 and 72 hours. Percentage viability in each arm was calculated relative to control cells normalised to 100%. Results are summarised in figure 4.2.

##### HB2

Incubation with FeSO<sub>4</sub> increased cell viability across all concentrations and time points ( $p < 0.05$ ). Haem loading had no effect at low concentrations but decreased cell viability at 40 $\mu$ M after 48 hours ( $p < 0.005$ ) and at 80 $\mu$ M after 48 ( $p = 0.0002$ ) and 72 ( $p = 0.004$ ) hours.

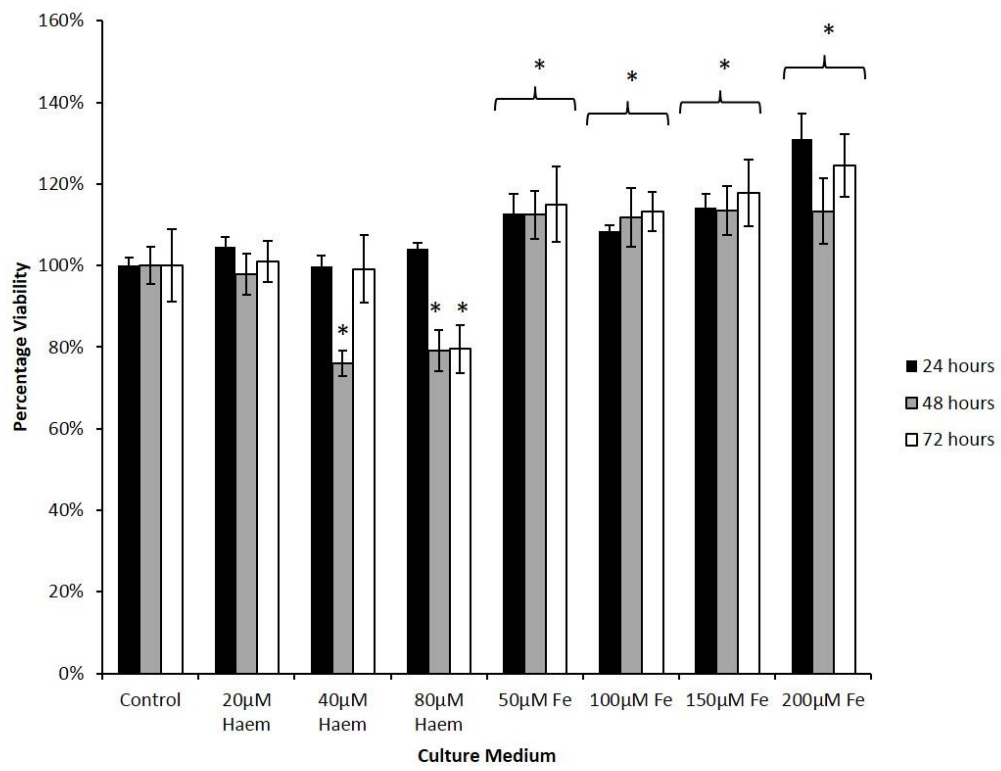
##### MCF7

150-200 $\mu$ M FeSO<sub>4</sub> induced increased viability across all 3 time intervals ( $p < 0.05$ ). 50-100 $\mu$ M FeSO<sub>4</sub> was only effective at 48 hours ( $p < 0.001$ ). 20 $\mu$ M haem increased viability from 24 to 72 hours ( $p < 0.02$ ). Higher haem concentrations were harmful to cells. Cells incubated in 40 $\mu$ M haem exhibited reduced cell viability at 72 hours ( $p < 0.001$ ); 80 $\mu$ M haem had a negative effect at both 48 and 72 hours ( $p < 0.002$ ).

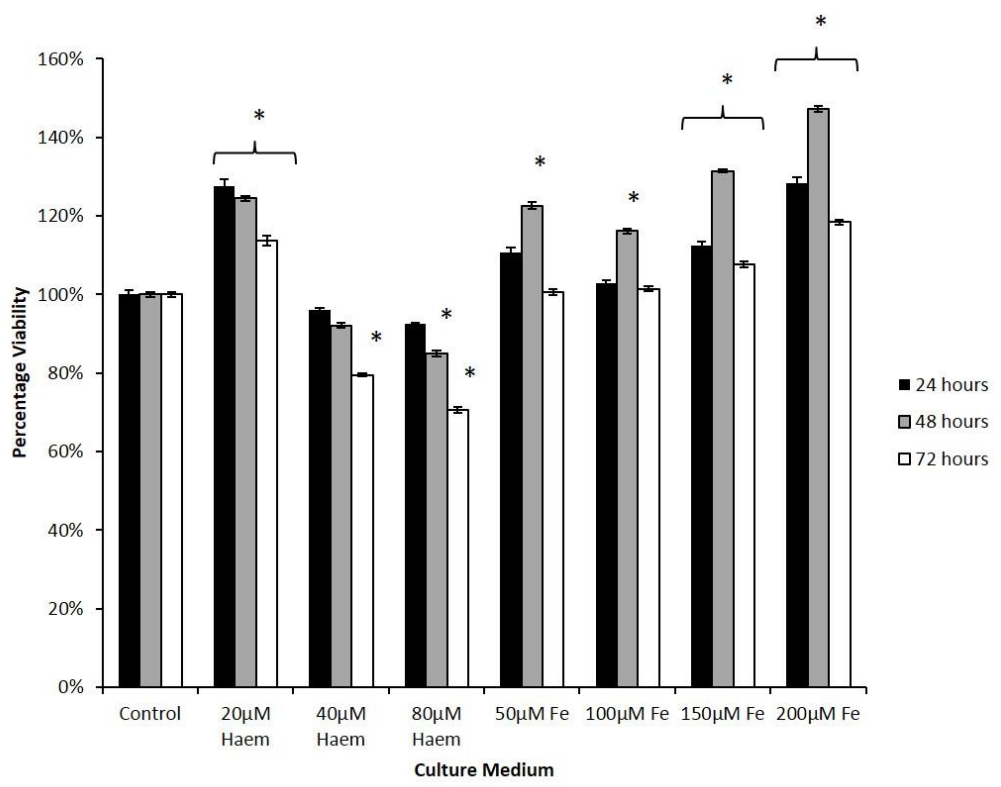
##### MDA-MB-231

Viability was significantly increased at all concentrations and time points with both FeSO<sub>4</sub> and haem loading ( $p < 0.05$ ). At higher haem concentrations the increase in viability relative to control was less profound, but remained statistically significant. FeSO<sub>4</sub> induced an increase in viability that became more marked as duration of incubation was prolonged.

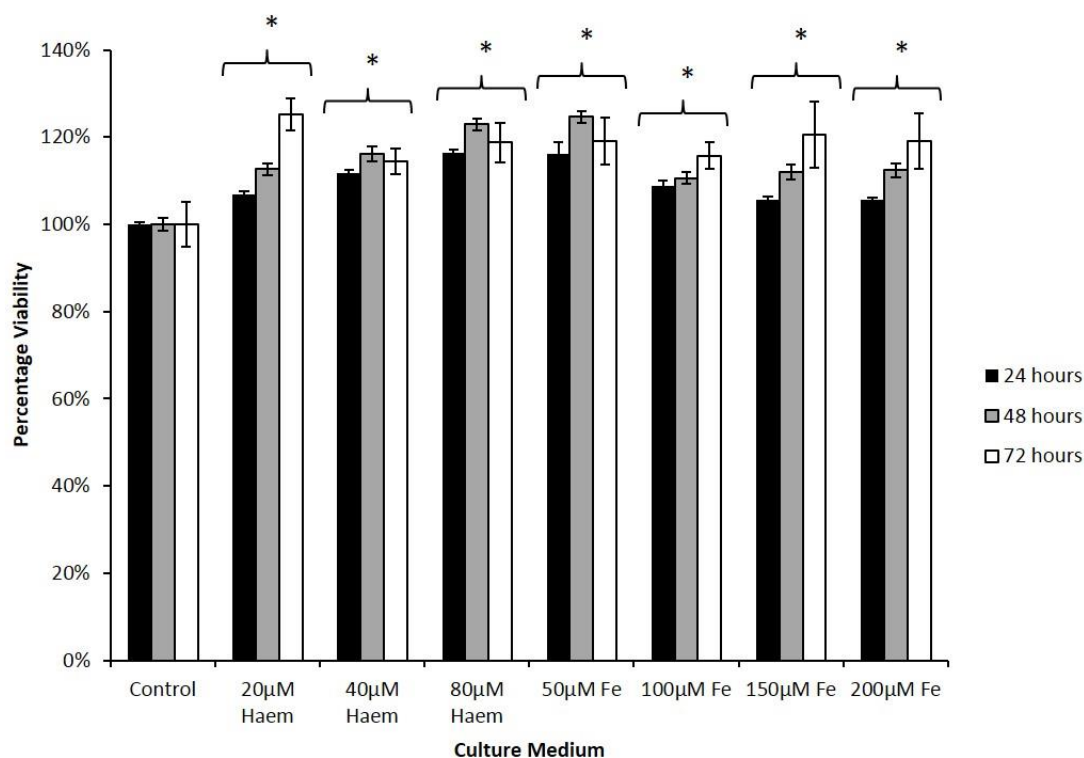
**HB2**



**MCF7**



## MDA-MB-231



**Figure 4.3 Changes in viability of benign and malignant mammary cells induced by iron and haem loading**

HB2 cells exhibit significantly up-regulated viability in response to  $\text{FeSO}_4$  loading at all concentrations and time points. Haem loading was largely ineffective but significantly deleterious at high concentrations. Viability of MCF7 cells was significantly increased following incubation with 20µM haem, but a significant inhibition of viability was seen at higher concentrations. Incubation with  $\text{FeSO}_4$  significantly stimulated viability of MCF7 cells at higher concentrations. MDA-MB-231 cells display universally up-regulated viability when incubated with  $\text{FeSO}_4$  or haem across all concentrations and time points. Statistical significance ( $p < 0.05$ ) is denoted by \* (student t-test). Results are expressed graphically as mean percentage viability (relative to control)  $\pm$  2SEM.



### **4.2.3 The Effects of Iron and Haem Loading on Cell Proliferation**

Changes in cellular proliferation induced by culturing cells with FeSO<sub>4</sub> and haem were measured using a BrdU assay as described in section 2.2.5.2. Concentrations and time points matched those used for the viability assays detailed in section 4.2.2. Percentage proliferation in each arm was calculated relative to control cells (unadulterated culture medium) normalised to 100%. Results are summarised in figure 4.3.

#### HB2

Incubating with haem for 24 hours at 20-80µM led to significantly increased cellular proliferation ( $p<0.02$ ). A significant increase was also observed at 48 hours at 80µM ( $p=0.004$ ). Incubation with FeSO<sub>4</sub> at 100-200µM led to significantly increased proliferation at 24 and 72 hours ( $p<0.05$ ).

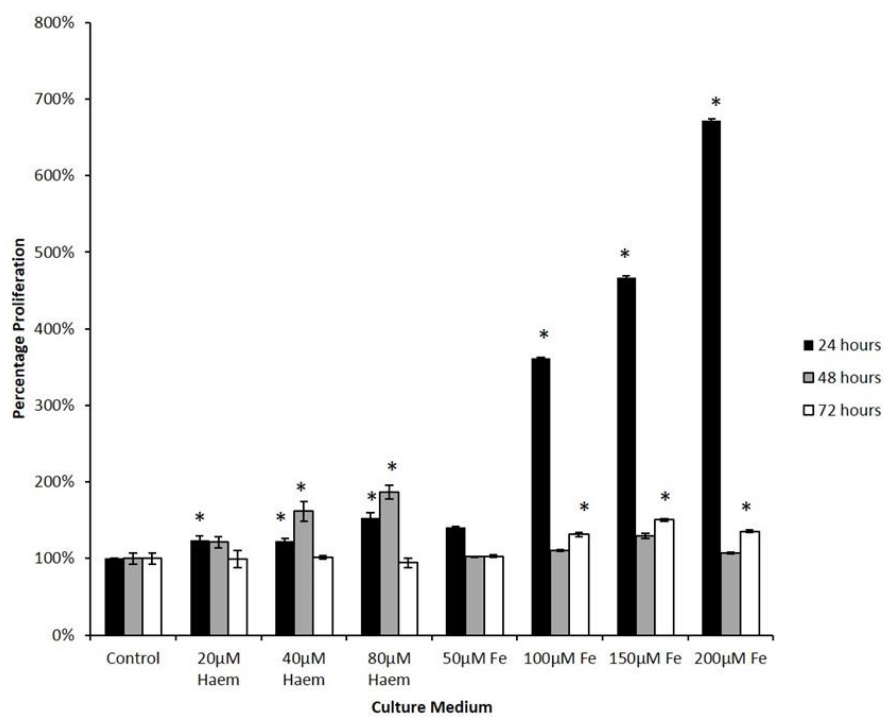
#### MCF7

MCF7 cells displayed significantly up-regulated proliferation at all haem concentrations across all time points, although at 72 hours the effects were less pronounced (all  $p<0.05$ ). FeSO<sub>4</sub> loading produced a significant increase in proliferation under all experimental conditions ( $p<0.04$ ) except following 72 hours incubation at 150 or 200µM where no changes were observed.

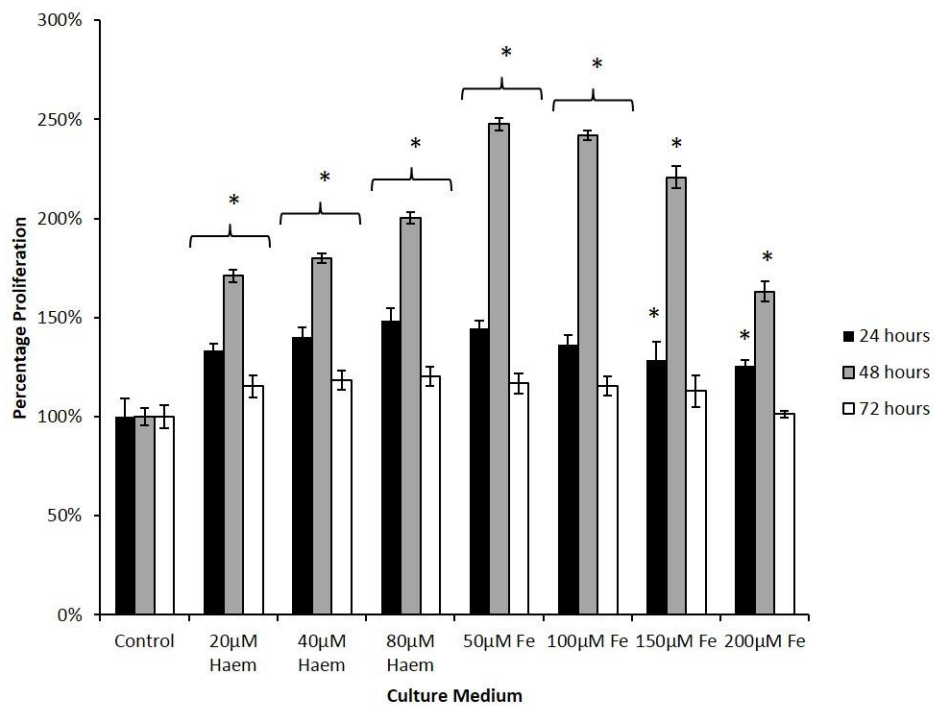
#### MDA-MB-231

After 24 hours, incubation with 20 and 40µM haem led to significantly increased proliferation as did 48 hours incubation at 20µM. 50-150µM FeSO<sub>4</sub> significantly up-regulated proliferation at all time points, as did 200µM at 24 and 72 hours (all  $p<0.05$ ).

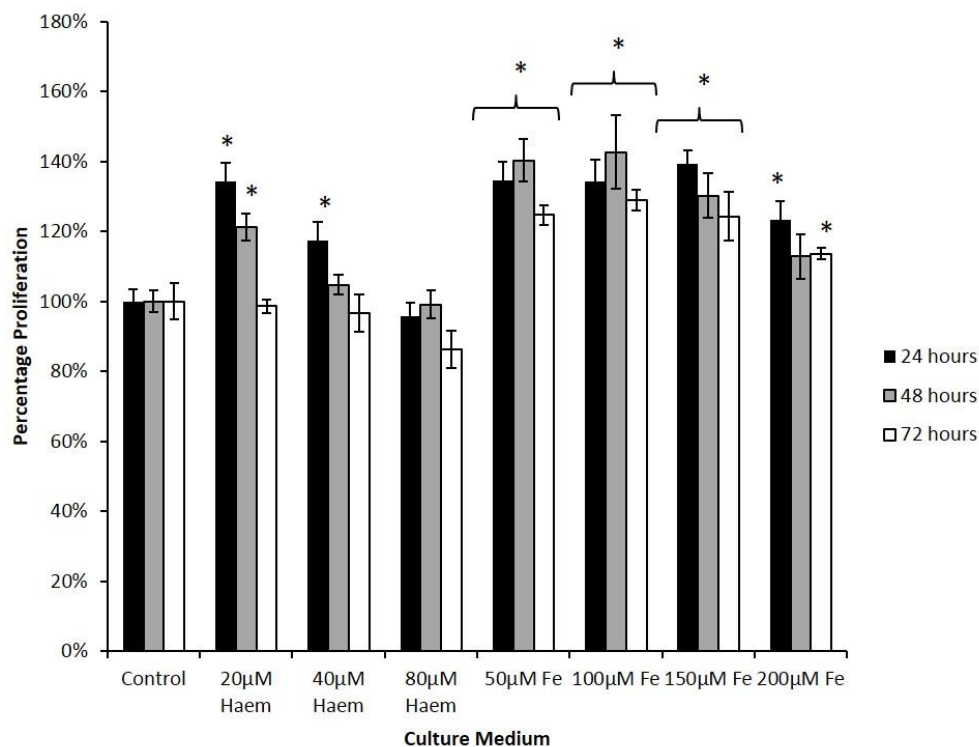
**HB2**



**MCF7**



## MDA-MB-231



**Figure 4.4 Changes in proliferation of benign and malignant mammary cells induced by iron and haem loading**

Proliferation of benign HB2 cells and malignant MCF7 and MDA-MB-231 cells was significantly increased after incubation with both haem and  $\text{FeSO}_4$  after varying amounts of time at varying concentrations. There were no inhibitory effects. Statistical significance is denoted by \* (student t-test). Results are expressed graphically as mean percentage proliferation (relative to control)  $\pm$  2SEM.

#### **4.2.4 The Effects of Iron Chelation on Cell Viability**

The experiments described in sections 4.2.2 and 4.2.3 indicated the concentrations of FeSO<sub>4</sub> and haem likely to induce the most profound changes in cell behaviour. Subsequent assays utilised these concentrations. ICL670A (deferasirox) was used to assess the effect of iron chelation on cell viability. HB2, MCF7 and MDA-MB-231 cells were incubated for 24 hours with 100µM FeSO<sub>4</sub> or 20µM haem, with and without 20µM ICL670A. Percentage viability was calculated relative to control cells normalised to 100%. Results are displayed in figure 4.4 and table 4-1.

##### **HB2**

FeSO<sub>4</sub> alone significantly increased HB2 cell viability with respect to control (p=0.038). This effect was reversed in the presence of ICL670A. No significant changes were observed in response to incubation with haem, with or without ICL670A. ICL670A alone also failed to provoke any significant change.

##### **MCF7**

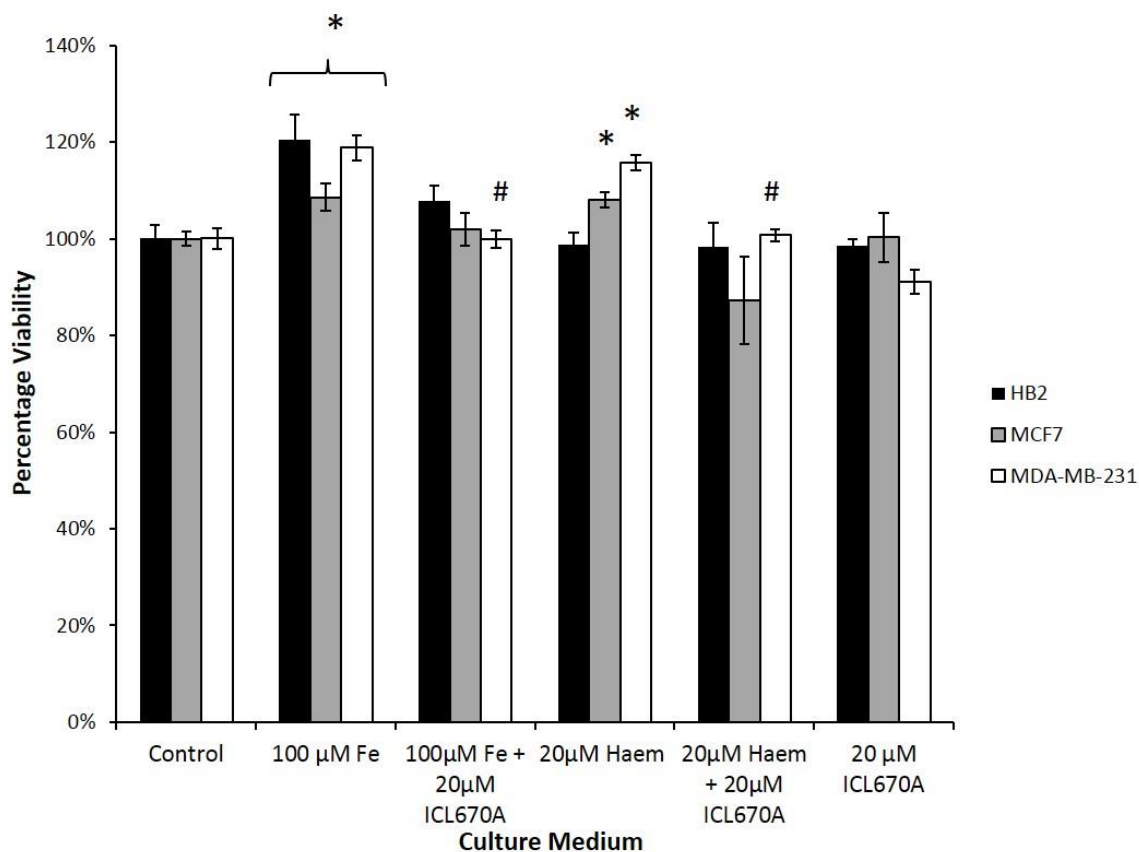
100µM FeSO<sub>4</sub> and 20µM haem significantly increased MCF7 viability over 24 hours (p<0.03). Introduction of ICL670A in addition to iron/haem abrogated this response and returned cellular viability to control levels. However p values did not indicate a significant difference between iron/haem alone and with ICL670A, implying incomplete resolution of the up-regulation in viability conferred by these reagents. ICL670A alone did not affect MCF7 viability.

##### **MDA-MB-231**

The increases in viability conferred by FeSO<sub>4</sub> and haem were maintained (p<0.04).

Subsequent ICL670A-mediated restoration of viability to levels approximate to control represented a statistically significant change relative to FeSO<sub>4</sub>/haem alone (p<0.03), implying

complete resolution of this up-regulation in viability. In isolation ICL670A did not alter viability significantly.



**Figure 4.5 The effects of iron chelation on benign and malignant mammary cell viability**

Cells were incubated with 100  $\mu$ M FeSO<sub>4</sub> and 20  $\mu$ M haem alone and in the presence of 20  $\mu$ M ICL670A, as well as 20  $\mu$ M ICL670A alone, for 24 hours. ICL670A proved capable of reversing the changes seen previously in response to FeSO<sub>4</sub>/haem, either wholly or partially, but had no significant effects on cell viability in isolation. \* denotes statistical significance relative to control and # that relative to chelator-free medium with other additives still present ( $p < 0.05$ , student t-test). Results are expressed graphically as mean percentage viability (relative to control)  $\pm$  2SEM.

	Cell Type		
	HB2	MCF7	MDA-MB-231
<b>Control</b>	100+/-2.8	100+/-1.5	100+/-2.7
<b>100µM Fe</b>	<b>120+/-5.1</b>	<b>109+/-2.7</b>	<b>119+/-2.6</b>
<b>100µM Fe + 20µM ICL670A</b>	108+/-3.0	102+/-3.4	100+/-1.8
<b>20µM Haem</b>	99+/-2.5	<b>108+/-1.7</b>	<b>116+/-1.6</b>
<b>20µM Haem + 20µM ICL670A</b>	98+/-5.0	87+/-9.0	101+/-1.2
<b>20µM ICL670A</b>	98+/-1.4	100+/-5.1	91+/-2.4

**Table 4-1 The effects of iron chelation on benign and malignant mammary cell viability**

This table summarises the data in figure 4.4. Values displayed are mean percentage viability of cells following 24 hours incubation with the reagents shown relative to control. Results are expressed as mean percentage viability +/- 2SEM. Significant values ( $p < 0.05$ ) relative to control are highlighted in bold print.

#### **4.2.5 The Effects of Iron Chelation on Cell Proliferation**

To assess the effect of iron chelation on cell proliferation, HB2, MCF7 and MDA-MB-231 cells were incubated for 24 hours in the presence of experimental media and a BrdU assay performed. Cells were exposed to control medium and media containing either 100 $\mu$ M FeSO<sub>4</sub> or 20 $\mu$ M haem, both with and without 20 $\mu$ M ICL670A. Percentage proliferation in each arm was calculated relative to control cells normalised to 100%. Results are displayed in figure 4.5 and table 4-2.

##### HB2

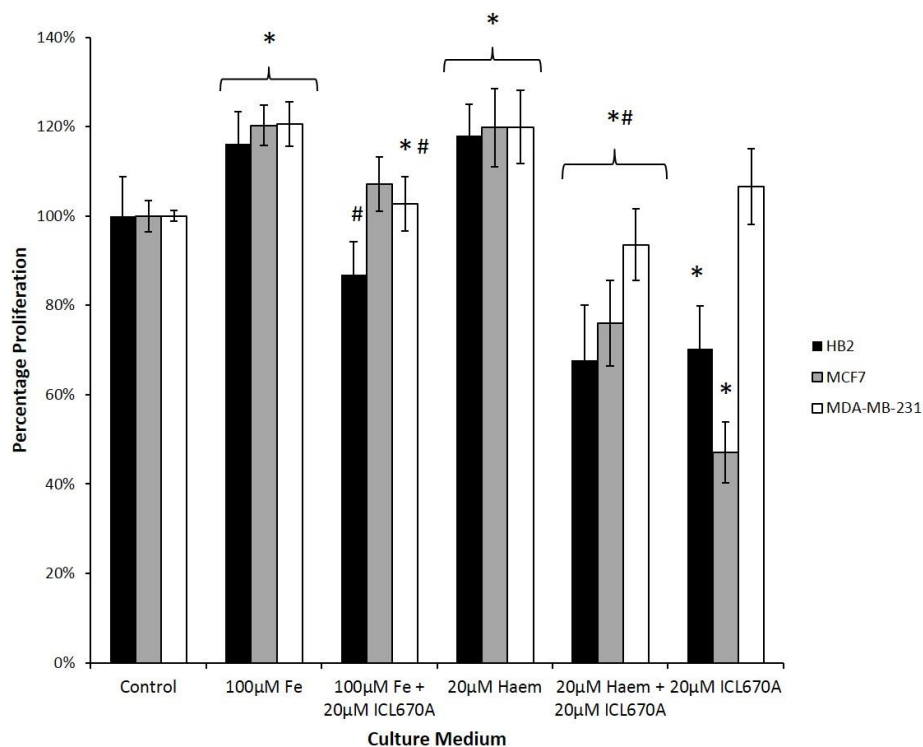
Incubation with FeSO<sub>4</sub> and haem induced statistically significant increases in cell proliferation ( $p < 0.04$ ). Co-culture with ICL670A reversed these effects, with a significant fall in proliferation relative to media containing iron/haem alone ( $p < 0.002$ ). ICL670A in isolation also had a significantly inhibitory effect on HB2 proliferation relative to control ( $p = 0.003$ ).

##### MCF7

Adding ICL670A to FeSO<sub>4</sub>- and haem-loaded media reversed the previously documented up-regulation in cell proliferation, although there was no statistically significant difference between the FeSO<sub>4</sub>/ICL670A population and cells incubated with FeSO<sub>4</sub> alone (implying incomplete abrogation). ICL670A induced a significant fall in proliferation when added to haem-containing media as compared to haem alone, and to control (both  $p = 0.003$ ). ICL670A alone significantly inhibited proliferation of MCF7 cells ( $p < 0.0005$ ).

##### MDA-MB-231

FeSO<sub>4</sub> and haem again led to significantly up-regulated proliferation ( $p < 0.05$ ). Adding ICL670A to these media induced significant falls in proliferation rates relative to both the stimulant-containing media and to control ( $p < 0.03$ ). ICL670A alone had no significant effect on proliferation relative to control media.



**Figure 4.6 The effects of iron chelation on benign and malignant mammary cell proliferation**

HB2, MCF7 and MDA-MB-231 cells were incubated for 24 hours with either 100μM FeSO<sub>4</sub> or 20μM haem, with and without 20μM ICL670A. A further population was incubated with 20μM ICL670A alone. The positive effects of FeSO<sub>4</sub> and haem on cell proliferation were once again observed. ICL670A was capable of wholly or partially abrogating these effects in both benign and malignant cells and also had a significant inhibitory effect on un-stimulated HB2 and MCF7 cells. \* denotes statistical significance relative to control and # that relative to chelator-free medium with other additives still present (p<0.05, student t-test). Results are expressed graphically as mean percentage proliferation (relative to control) +/- 2SEM.



	Cell Type		
	HB2	MCF7	MDA-MB-231
<b>Control</b>	100+/-8.8	100+/-3.5	100+/-1.1
<b>100µM Fe</b>	116+/-7.2	<b>120+/-4.6</b>	<b>121+/-5.0</b>
<b>100µM Fe + 20µM ICL670A</b>	87+/-7.3	107+/-6.1	<b>103+/-6.1</b>
<b>20µM Haem</b>	118+/-7.0	<b>120+/-8.8</b>	<b>120+/-8.3</b>
<b>20µM Haem + 20µM ICL670A</b>	<b>68+/-12.3</b>	<b>76+/-9.6</b>	<b>94+/-8.0</b>
<b>20µM ICL670A</b>	<b>70+/-9.7</b>	<b>47+/-6.8</b>	107+/-8.5

**Table 4-2 The effects of iron chelation on benign and malignant mammary cell proliferation**

This table summarises the data in figure 4.5. Values displayed are mean percentage proliferation of cells following 24 hours incubation with the reagents shown relative to control. Results are expressed as mean percentage proliferation +/- 2SEM. Significant values ( $p < 0.05$ ) relative to control are highlighted in bold print.

#### **4.2.6 The Effects of Iron and Haem Loading on Anchorage-Independent Growth**

The effects of 100 $\mu$ M FeSO<sub>4</sub>, 20 $\mu$ M haem and 20 $\mu$ M ICL670A on anchorage-independent growth were measured in MCF7 and MDA-MB-231 cells as described in section 2.2.5.3. HB2 cells are incapable of forming colonies and were not subjected to this assay.

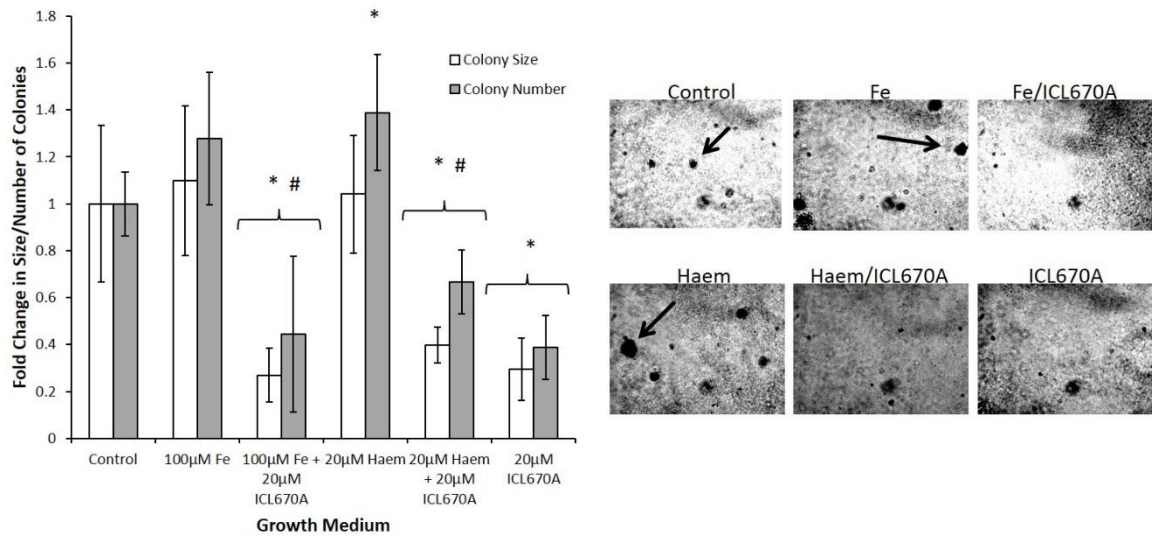
##### MCF7

FeSO<sub>4</sub> did not induce a significant change in mean size or number of colonies formed relative to control agar. Haem had no effect on mean colony size but significantly increased numbers of colonies formed ( $p=0.032$ ). 20 $\mu$ M ICL670A had profound effects on colony formation, reversing the haem-induced increase in colony numbers ( $p=0.002$ ) and significantly inhibiting the size and number of colonies formed whether or not iron or haem were also present (all  $p<0.03$ ). Results are shown in figure 4.6 and table 4-3.

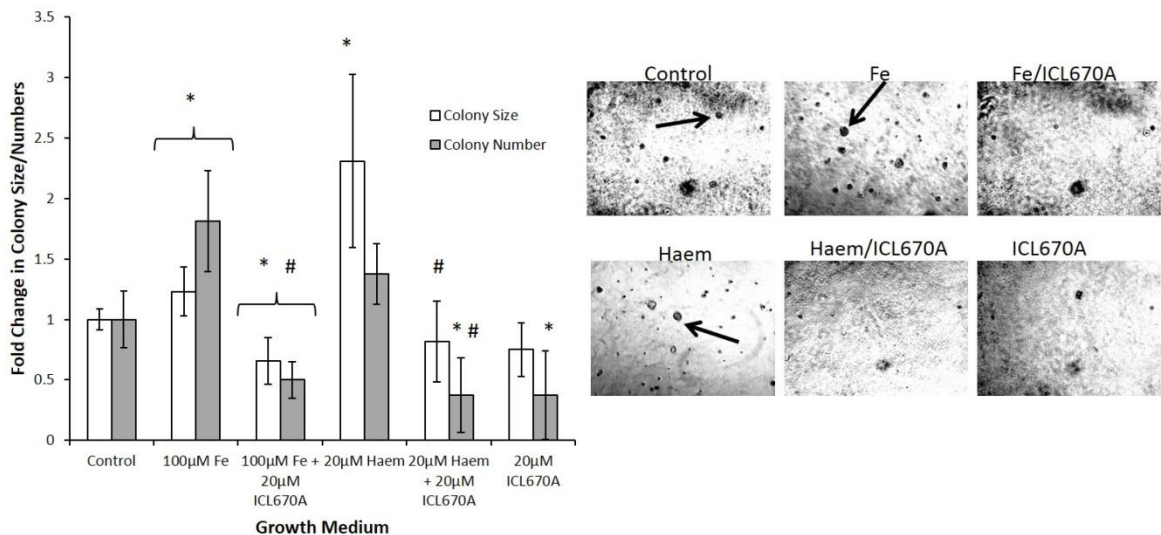
##### MDA-MB-231

FeSO<sub>4</sub> significantly up-regulated the mean number ( $p=0.013$ ) and size ( $p=0.044$ ) of colonies formed. These changes were reversed by ICL670A with a significant down-regulation of both size and number (both  $p=0.009$ ) relative to control and to FeSO<sub>4</sub> alone (both  $p<0.002$ ). Haem significantly up-regulated mean colony size ( $p=0.0015$ ); this effect was reversed by ICL670A ( $p=0.0008$ ). In isolation ICL670A significantly down-regulated colony numbers relative to control ( $p=0.024$ ) but had no significant effect on colony size. Results are displayed in figure 4.6 and table 4-3.

## MCF7



## MDA-MB-231



**Figure 4.7 The effects of FeSO<sub>4</sub>, haem and iron chelation on anchorage-independent growth of malignant mammary cells**

MCF7 and MDA-MB-231 cells were seeded in agar gels containing either 100µM FeSO<sub>4</sub> or 20µM haem, with and without 20µM ICL670A, and 20µM ICL670A alone. Colonies were counted and measured at 2 weeks. \* denotes significance relative to control and # that relative to chelator-free medium with other additives still present ( $p < 0.05$ , student t-test). Results are expressed as mean fold change in colony size/number relative to control  $\pm$  2SEM.

Cell Type	Agar Additives	Mean Fold Change in Colony Number	Mean Fold Change in Colony Size
<b>MCF7</b>	100µM FeSO <sub>4</sub>	1.28+/-0.28	1.1+/-0.32
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>0.44+/-0.33</b>	<b>0.27+/-0.11</b>
	20µM Haem	<b>1.39+/-0.25</b>	1.04+/-0.25
	20µM Haem + 20µM ICL670A	<b>0.67+/-0.14</b>	<b>0.4+/-0.07</b>
	20µM ICL670A	<b>0.39+/-0.14</b>	<b>0.29+/-0.13</b>
<b>MDA-MB-231</b>	100µM FeSO <sub>4</sub>	<b>1.81+/-0.41</b>	<b>1.23+/-0.2</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>0.5+/-0.15</b>	<b>0.66+/-0.19</b>
	20µM Haem	1.38+/-0.25	<b>2.31+/-0.72</b>
	20µM Haem + 20µM ICL670A	<b>0.38+/-0.31</b>	0.82+/-0.34
	20µM ICL670A	<b>0.38+/-0.36</b>	0.75+/-0.22

**Table 4-3 Changes in colony formation after treatment with FeSO<sub>4</sub>, haem and ICL670A**

The table summarizes mean fold changes in colony size and number following incubation of cells in agar gels containing 100µM FeSO<sub>4</sub> or 20µM haem, with and without 20µM ICL670A, and 20µM ICL670A alone. Results are expressed as mean fold change in size/number of colonies relative to control medium +/- 2SEM. Significant values (p<0.05) relative to control are highlighted in bold print.

#### **4.2.7 The Effects of Iron on Cell Migration**

Scratch assays were used to evaluate the effects of FeSO<sub>4</sub>, haem and the chelator ICL670A on the ability of benign and malignant mammary cells to migrate across a wound created in a confluent cell monolayer. Each cell line was exposed to control medium and media containing either 100µM FeSO<sub>4</sub> or 20µM haem, both with and without 20µM ICL670A.

##### HB2

There was no increase in migratory capacity following incubation with iron or haem. Incubating cells with ICL670A alone and in conjunction with FeSO<sub>4</sub> led to a significant fall in rate of migration (both  $p < 0.01$ ). Results are displayed in figures 4.7 and 4.8 and table 4-2.

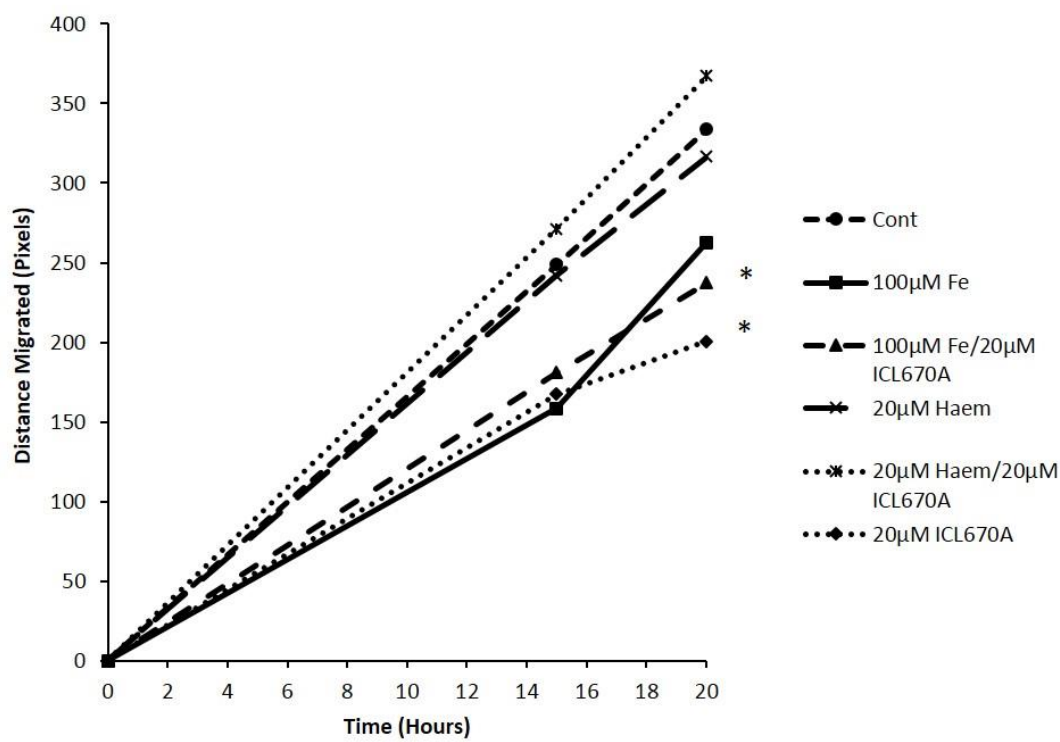
##### MCF7

FeSO<sub>4</sub> ( $p=0.037$ ) and haem ( $p=0.019$ ) significantly up-regulated migration of MCF7 cells. This was reversed following addition of ICL670A to stimulant media, although this reversal was only significant relative to the stimulant medium alone in the case of FeSO<sub>4</sub> ( $p=0.024$ ). In isolation ICL670A had no significant effect on cell migration relative to control. Results are displayed in figures 4.7 and 4.8 and table 4-2.

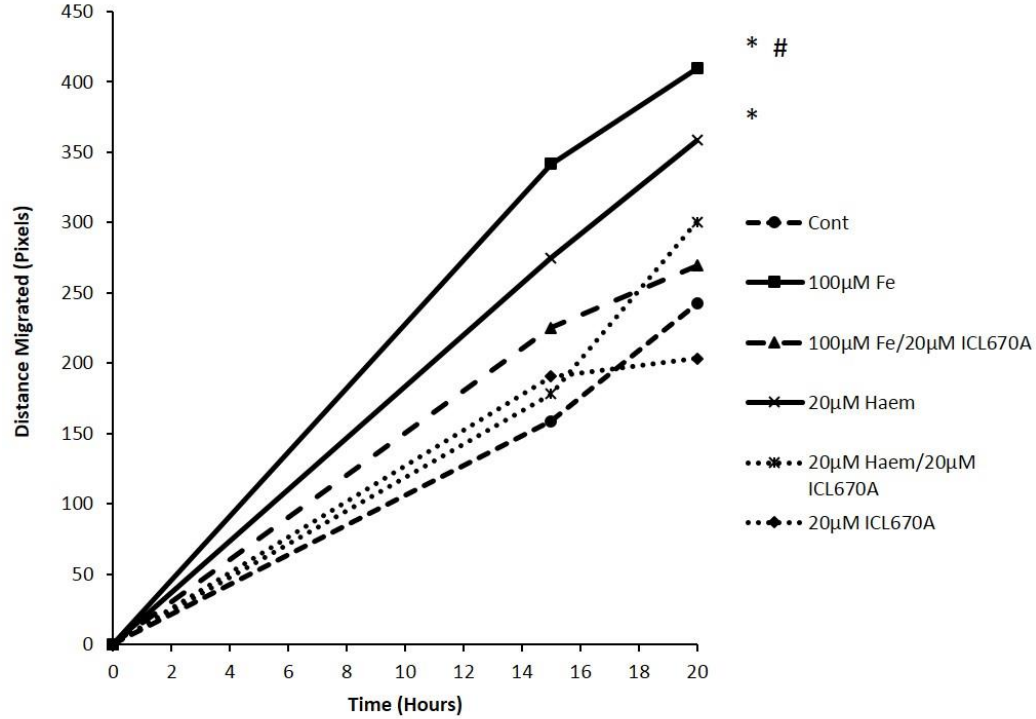
##### MDA-MB-231

FeSO<sub>4</sub> and haem demonstrated a significantly positive effect on cell migration (both  $p=0.049$ ), although the subsequent down-regulation following addition of ICL670A failed to reach significance in either the FeSO<sub>4</sub>- or haem-loaded cells. ICL670A alone had no effect relative to control. Results are displayed in figures 4.7 and 4.8 and table 4-2.

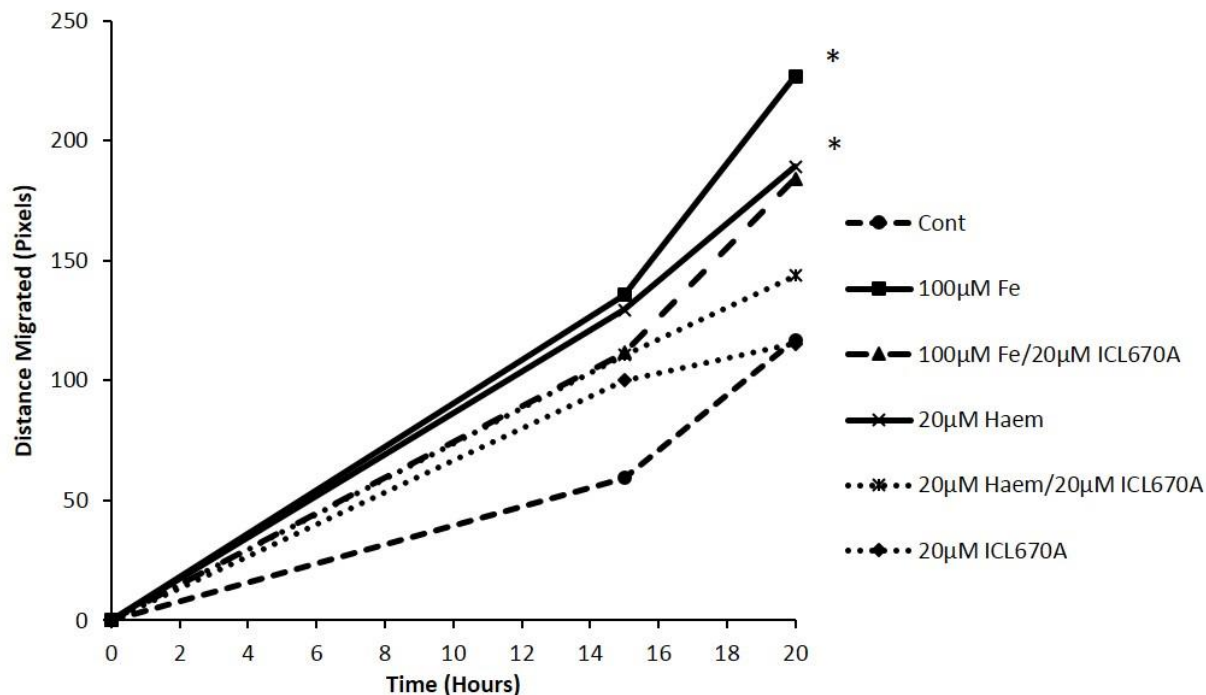
HB2



MCF7



## MDA-MB-231

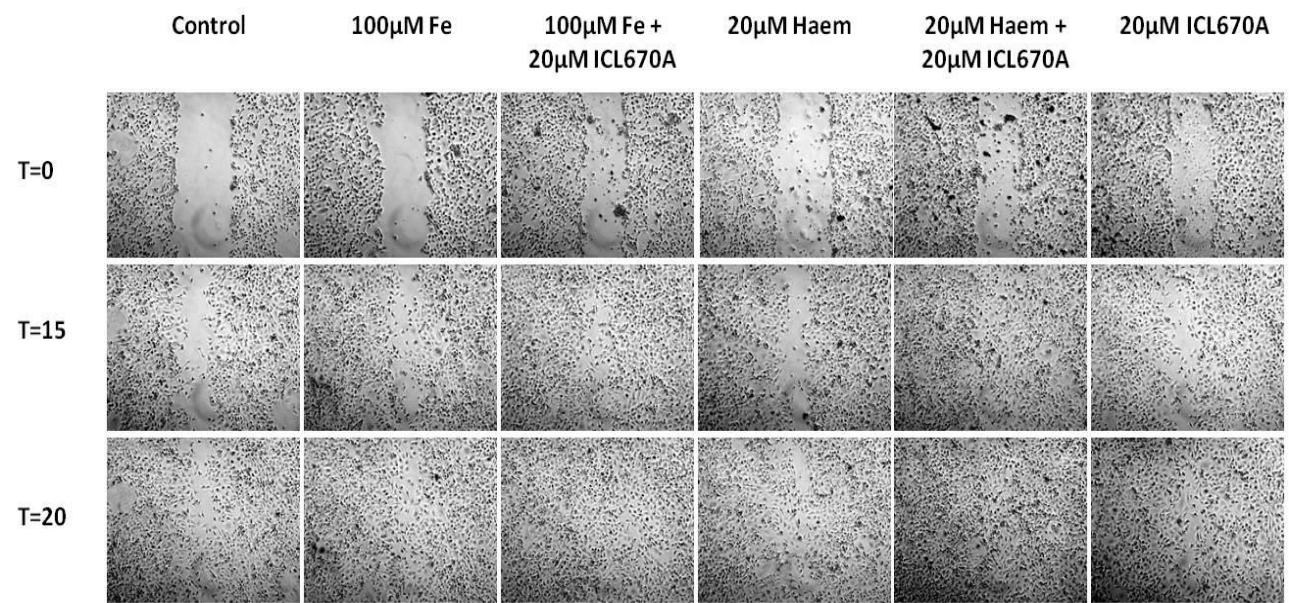


**Figure 4.8 The effects  $\text{FeSO}_4$ , haem and iron chelation on migration of benign and malignant mammary cells**

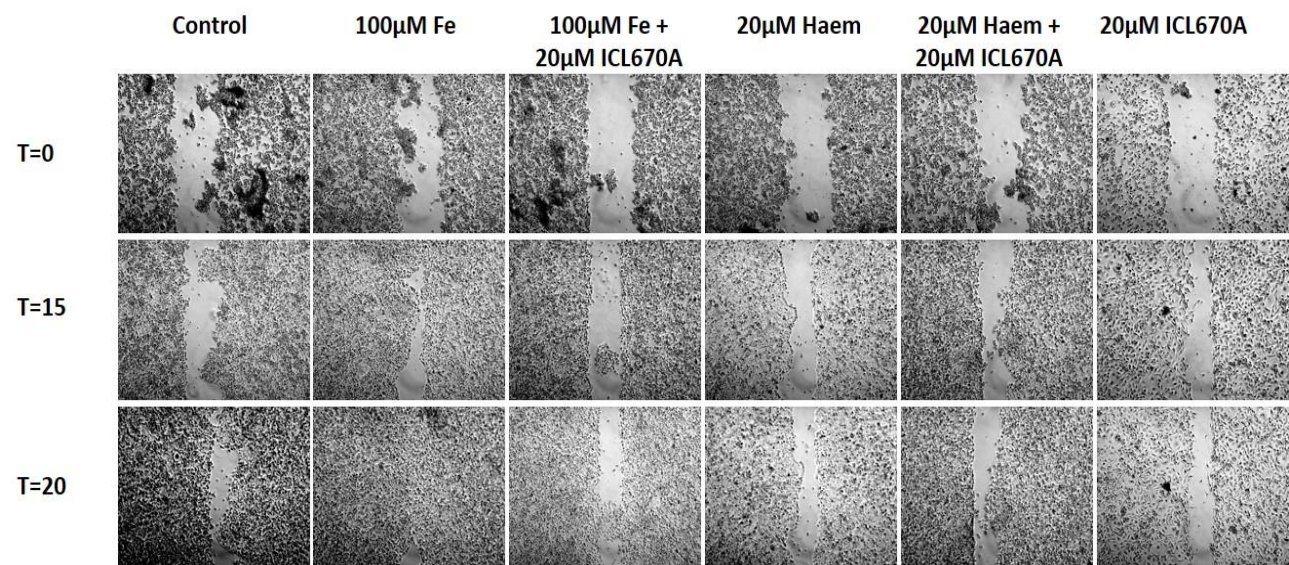
Scratched monolayers of HB2, MCF7 and MDA-MB-231 cells were incubated for 20 hours with media containing  $100\mu\text{M}$   $\text{FeSO}_4$  or  $20\mu\text{M}$  haem, with and without  $20\mu\text{M}$  ICL670A. A further population was incubated with  $20\mu\text{M}$  ICL670A alone. Results are expressed as mean distance migrated  $\pm$  2SEM. \* denotes statistical significance relative to control and # that relative to chelator-free medium with other additives still present ( $p < 0.05$ , student t-test).



**HB2**

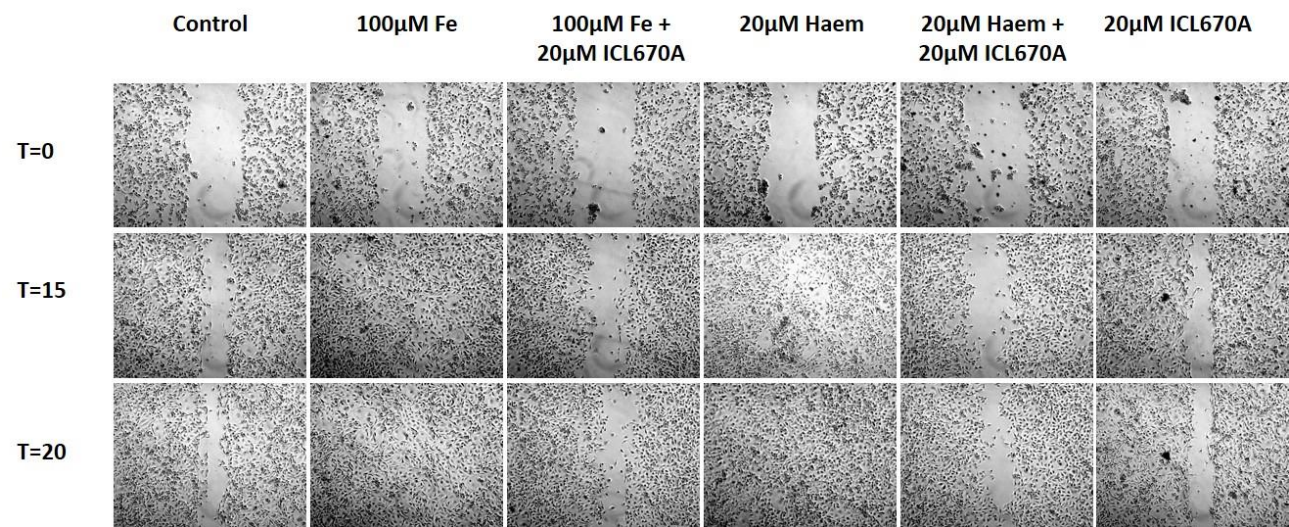


**MCF7**





**MDA-MB-231**



**Figure 4.9 Rates of mammary cell migration in the presence of iron, haem and ICL670A**

HB2, MCF7 and MDA-MB-231 cells were incubated with 100µM FeSO<sub>4</sub> or 20µM haem, with and without 20µM ICL670A. A further population was incubated with 20µM ICL670A alone. Photomicrographs were taken of wounds made in the cell monolayers as time progressed and distance travelled by cells across the wound measured for comparison with cells in control medium. Results are presented in table 4-2 and figure 4.7.

Cell Type	Composition of Culture Medium	Mean Distance Migrated (pixels) over 20 Hours
<b>HB2</b>	Control	333.8+/-39.3
	100µM FeSO <sub>4</sub>	262.8+/-79.8
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>237.5+/-21.8</b>
	20µM Haem	316.8+/-59.6
	20µM Haem + 20µM ICL670A	367.2+/-22
	20µM ICL670A	<b>200.8+/-32.6</b>
<b>MCF7</b>	Control	242.5+/-56.7
	100µM FeSO <sub>4</sub>	<b>409.8+/-15.5</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	269.5+/-68.9
	20µM Haem	<b>358.5+/-41.3</b>
	20µM Haem + 20µM ICL670A	300.5+/-44.9
	20µM ICL670A	203.3+/-34.1
<b>MDA-MB-231</b>	Control	116.8+/-46.7
	100µM FeSO <sub>4</sub>	<b>226.8+/-72.4</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	184+/-13.9
	20µM Haem	<b>189+/-12.7</b>
	20µM Haem + 20µM ICL670A	143.8+/-62.2
	20µM ICL670A	115.3+/-34

**Table 4-4 Changes in cell migration following treatment with FeSO<sub>4</sub>, haem and ICL670A**

Wound-healing assays were performed over a 20 hour period in the presence of combinations of FeSO<sub>4</sub>, haem and ICL670A as shown. Results are expressed as mean distance migrated +/- 2SEM. Significant values (p<0.05) relative to control are highlighted in bold print.

#### **4.2.8 The Effects of FeSO<sub>4</sub>, Haem and Iron Chelation on the Invasive Capacity of Breast Cells**

The effects of iron, haem and iron chelation on cellular invasion were assessed using a matrigel invasion assay. Each cell line was exposed to control medium and media containing either 100µM FeSO<sub>4</sub> or 20µM haem, both with and without 20µM ICL670A.

##### HB2

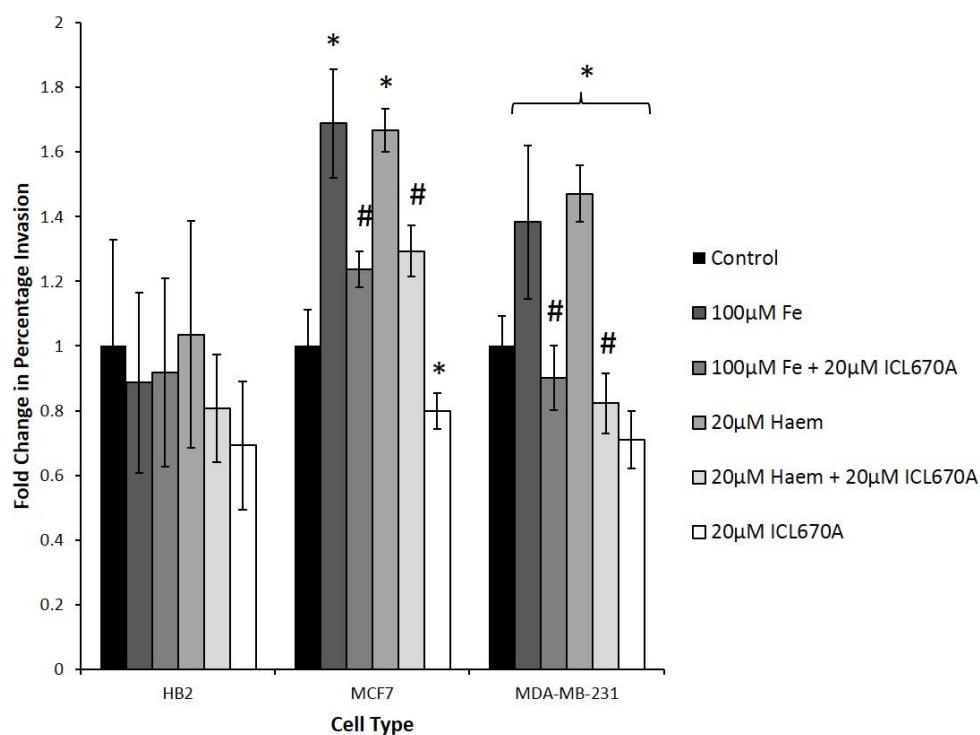
No effect on invasive capacity of benign HB2 cells was seen with either FeSO<sub>4</sub> or haem. Adding ICL670A to control and stimulant media had no significant effect. Results are displayed in figure 4.9 and table 4-5.

##### MCF7

FeSO<sub>4</sub> (p=0.018) and haem (p=0.001) significantly increased the invasive capacity of MCF7 cells. Co-culture with ICL670A reversed this change, causing a significant drop in invasive capability relative to cells incubated with FeSO<sub>4</sub> (p=0.01) or haem (p<0.001) alone, although there was no significant difference relative to control cells. ICL670A alone had a significant negative effect on invasiveness of MCF7 cells relative to control (p=0.012). Results are displayed in figure 4.9 and table 4-5.

##### MDA-MB-231

Invasive capacity of MDA-MB-231 cells was significantly increased by adding FeSO<sub>4</sub> (p=0.004) or haem (p<0.001) to incubation medium. Adding ICL670A to stimulant media nullified this change, causing a significant fold decrease in percentage invasion of FeSO<sub>4</sub> and haem loaded cells relative to both stimulated and control cells (all p<0.02). ICL670A alone significantly inhibited invasion relative to control (p=0.012). Results are displayed in figure 4.9 and table 4-5.



**Figure 4.10 The effects of FeSO<sub>4</sub>, haem and iron chelation on invasive capacity of benign and malignant mammary cells**

Cells were left in transwell plates for 24 hours in the presence of combinations of FeSO<sub>4</sub>, haem and ICL670A as shown. Percentage invasion through a matrigel layer was measured and results expressed as mean fold change relative to control  $\pm$  2SEM. \*denotes statistical significance relative to control and # that relative to chelator-free medium with other additives still present ( $p < 0.05$ , student t-test). Results are summarized in table 4-5.

Cell Type	Composition of Culture Medium	Mean Fold Change in Percentage Invasion
<b>HB2</b>	Control	1+/-0.33
	100μM FeSO <sub>4</sub>	0.89+/-0.28
	100μM FeSO <sub>4</sub> + 20μM ICL670A	0.92+/-0.29
	20μM Haem	1.04+/-0.35
	20μM Haem + 20μM ICL670A	0.81+/-0.17
	20μM ICL670A	0.69+/-0.2
<b>MCF7</b>	Control	1+/-0.11
	100μM FeSO <sub>4</sub>	<b>1.69+/-0.17</b>
	100μM FeSO <sub>4</sub> + 20μM ICL670A	1.24+/-0.06
	20μM Haem	<b>1.67+/-0.07</b>
	20μM Haem + 20μM ICL670A	1.29+/-0.08
	20μM ICL670A	<b>0.8+/-0.06</b>
<b>MDA-MB-231</b>	Control	1+/-0.09
	100μM FeSO <sub>4</sub>	<b>1.38+/-0.24</b>
	100μM FeSO <sub>4</sub> + 20μM ICL670A	<b>0.9+/-0.1</b>
	20μM Haem	<b>1.47+/-0.09</b>
	20μM Haem + 20μM ICL670A	<b>0.8+/-0.09</b>
	20μM ICL670A	<b>0.71+/-0.09</b>

**Table 4-5 Changes in cell invasion following treatment with iron, haem and ICL670A**

Invasion assays were performed over 24 hours in the presence of stimulant and chelating reagents as shown. Results are expressed as mean fold change relative to control +/- 2SEM. Significant values (p<0.05) relative to control are highlighted in bold print.

		100µM FeSO4	100µM FeSO4 + 20µM ICL670A	20µM Haem	20µM Haem + 20µM ICL670A	20µM ICL670A
<b>HB2</b>	Viability	↑	→	→	→	→
	Proliferation	↑	→	↑	↓	↓
	Colony Formation	-	-	-	-	-
	Migration	→	↓	→	→	↓
	Invasion	→	→	→	→	→
<b>MCF7</b>	Viability	↑	→	↑	→	→
	Proliferation	↑	→	↑	↓	↓
	Colony Formation	Size	→	↓	→	↓
		No.	→	↓	↑	↓
	Migration	↑	→	↑	→	→
	Invasion	↑	→	↑	→	↓
<b>MDA-MB-231</b>	Viability	↑	→	↑	→	→
	Proliferation	↑	↑	↑	↓	→
	Colony Formation	Size	↑	↓	↑	→
		No.	↑	↓	→	↓
	Migration	↑	→	↑	→	→
	Invasion	↑	↓	↑	↓	↓

**Table 4-6 A summary of the effects of iron, haem and ICL670A on cell phenotype**

This table provides an overview of the overall impact of iron, haem and ICL670A, alone and in combination, on various aspects of cellular phenotype. Benign HB2 and malignant MCF7 and MDA-MB-231 cells were studied. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→). HB2 cells were not subjected to a colony-forming assay.

### **4.3 The Effects of Exogenous Iron, Haem and Iron Chelators on the Iron Transport Mechanism in Vitro**

To determine the effects of modulating exogenous iron levels on benign and malignant mammary cell expression of the iron and haem transport proteins and their mRNA, HB2 cells were compared to the malignant MDA-MB-231 cell line.

Cells were incubated for 24 hours in the presence of control medium or media containing 100 $\mu$ M FeSO<sub>4</sub> or 20 $\mu$ M hemin, both with and without 20 $\mu$ M ICL670A. Levels of the iron and haem transporters and their mRNA were evaluated following exposure. Results are reported throughout as expression of protein or mRNA under experimental conditions relative to that in control media which has been normalised to a level of 1.

### **4.3.1 Changes in Expression of Iron and Haem Transport Proteins in Response to Iron and Chelators**

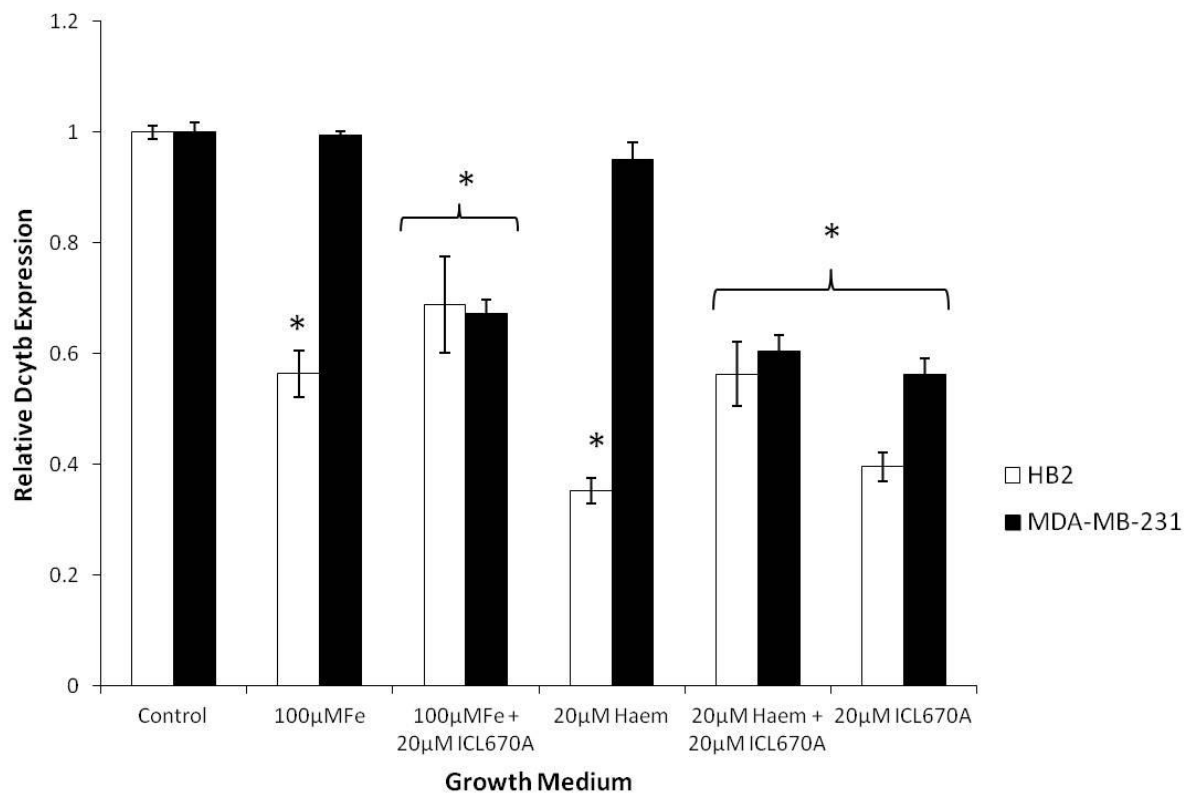
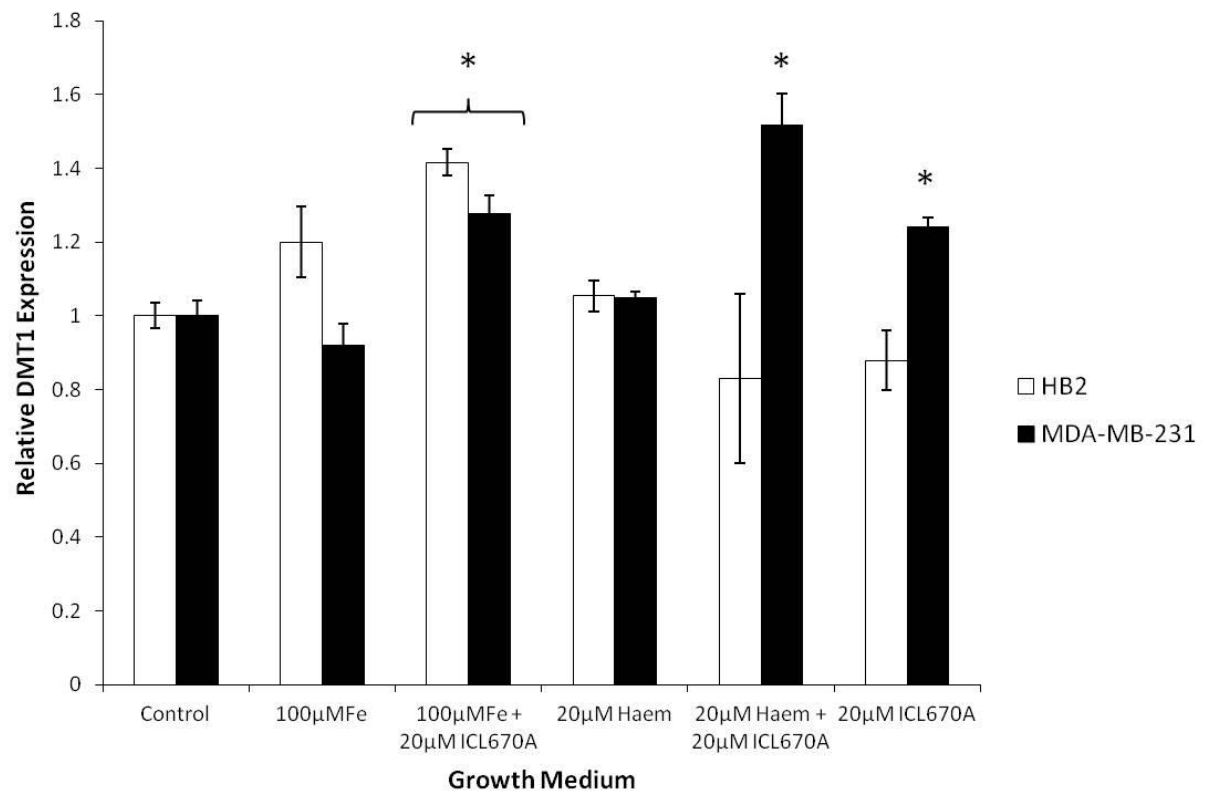
#### *4.3.1.1 Iron Import Proteins*

Expression of Dcytb was significantly reduced across all experimental media relative to control in HB2 cells ( $p < 0.02$  throughout). Although the introduction of ICL670A partially ameliorated this effect, levels did not return to normal. There was no significant change in expression of Dcytb in MDA-MB-231 cells upon introduction of either iron or haem. The presence of a chelator had a negative effect on Dcytb expression in the malignant line, both with and without iron or haem in the culture medium ( $p < 0.0002$  throughout).

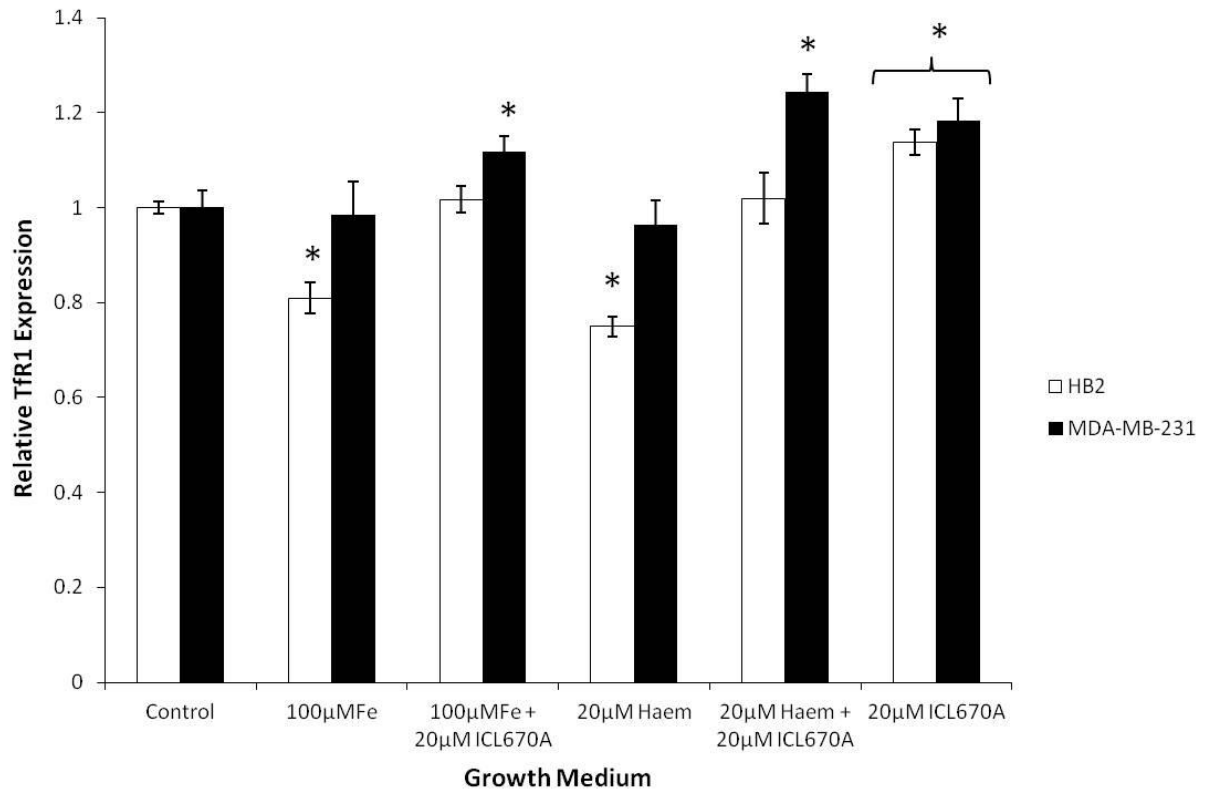
DMT1 expression levels were unchanged in both HB2 and MDA-MB-231 cells in the presence of either iron or haem. The mixture of iron and ICL670A induced a significant up-regulation in DMT1 in both cell lines (both  $p < 0.002$ ). DMT1 was also up-regulated to a significant extent in the malignant line following culture with haem and ICL670A, and with ICL670A alone (both  $p < 0.002$ ).

TfR1 levels were significantly up-regulated in the MDA-MB-231 cells following culture with ICL670A in the presence and absence of iron and haem (all  $p < 0.009$ ), but were unchanged by culturing with iron or haem alone. HB2 cells exhibited an appropriate down-regulation of TfR1 expression after culture with iron or haem (both  $p < 0.004$ ), which was eliminated by the addition of ICL670A.



**A****B**

C



**Figure 4.11 Relative expression of the iron import proteins in benign and malignant breast cells following exposure to iron and chelators**

Protein expression of Dcytb (A), DMT1 (B) and TfR1 (C) was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

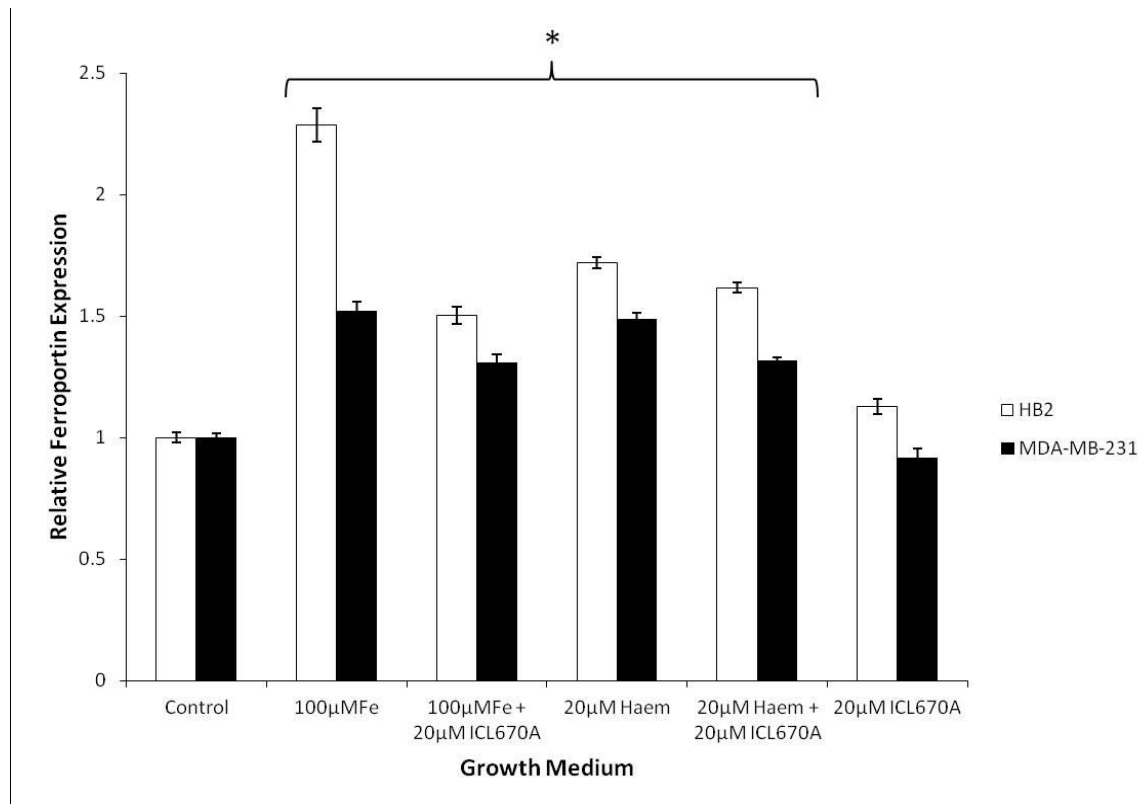
Cell Type	Experimental Medium	Relative Dcytb Expression	Relative DMT1 Expression	Relative TfR1 Expression
<b>HB2</b>	Control	1+/-0.01	1+/-0.03	1+/-0.01
	100µM FeSO <sub>4</sub>	<b>0.56+/-0.04</b>	1.12+/-0.1	<b>0.81+/-0.03</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>0.69+/-0.09</b>	<b>1.42+/-0.04</b>	1.02+/-0.03
	20µM Haem	<b>0.35+/-0.02</b>	1.05+/-0.04	<b>0.75+/-0.02</b>
	20µM Haem + 20µM ICL670A	<b>0.56+/-0.06</b>	0.83+/-0.2	1.02+/-0.05
	20µM ICL670A	<b>0.4+/-0.03</b>	0.88+/-0.08	<b>1.14+/-0.03</b>
<b>MDA-MB-231</b>	Control	1+/-0.02	1+/-0.04	1+/-0.04
	100µM FeSO <sub>4</sub>	0.99+/-0.01	0.92+/-0.06	0.99+/-0.07
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>0.67+/-0.02</b>	<b>1.28+/-0.05</b>	<b>1.12+/-0.03</b>
	20µM Haem	0.95+/-0.03	1.05+/-0.02	0.96+/-0.05
	20µM Haem + 20µM ICL670A	<b>0.61+/-0.03</b>	<b>1.52+/-0.08</b>	<b>1.24+/-0.04</b>
	20µM ICL670A	<b>0.56+/-0.03</b>	<b>1.24+/-0.02</b>	<b>1.18+/-0.05</b>

**Table 4-7 Relative expression of the iron import proteins in benign and malignant breast cells following exposure to iron and chelators**

Protein expression of Dcytb, DMT1 and TfR1 was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.1.2 Iron Export Proteins

Expression of ferroportin was significantly increased in both benign HB2 and malignant MDA-MB-231 cells following culture with iron or haem, with and without ICL670A (all  $p < 0.006$ ). Neither cell type displayed any change in expression after culture with ICL670A alone.



**Figure 4.12 Relative ferroportin expression in benign and malignant breast cells following exposure to iron and chelators**

Expression of ferroportin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

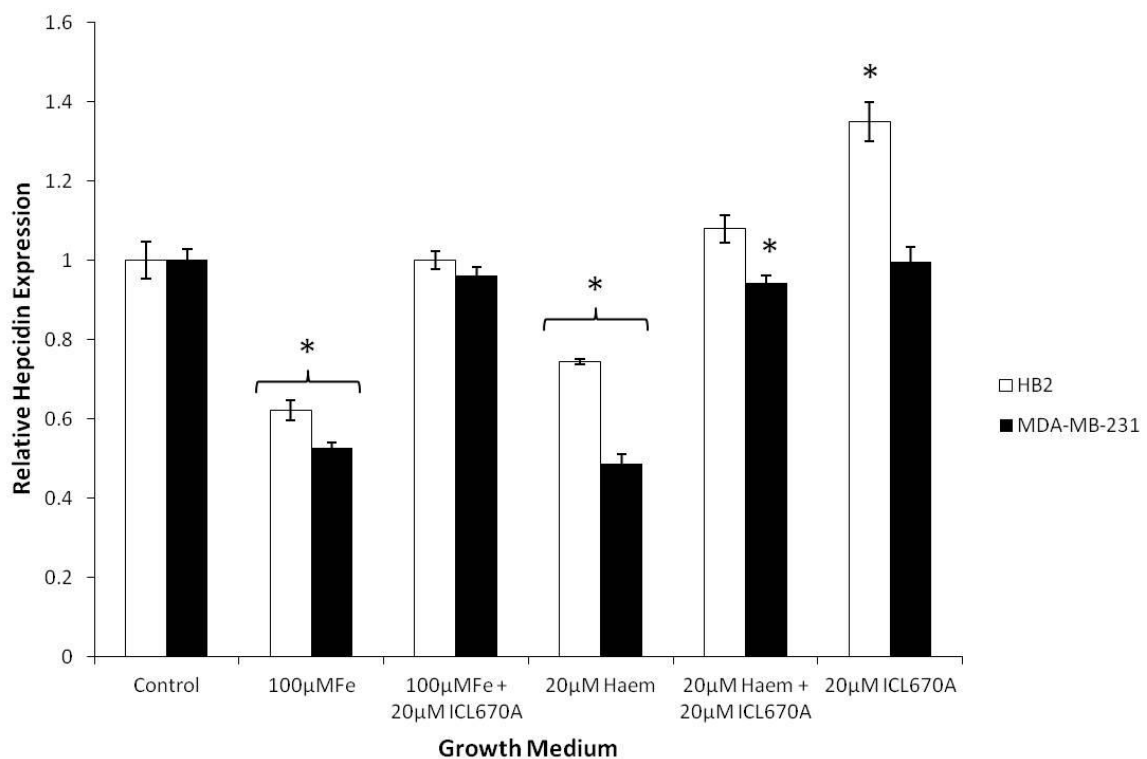
Cell Type	Culture Medium	Relative Ferroportin Expression
<b>HB2</b>	Control	1+/-0.02
	100µM FeSO <sub>4</sub>	<b>2.28+/-0.07</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>1.5+/-0.04</b>
	20µM Haem	<b>1.72+/-0.02</b>
	20µM Haem + 20µM ICL670A	<b>1.62+/-0.02</b>
	20µM ICL670A	1.13+/-0.03
<b>MDA-MB-231</b>	Control	1+/-0.02
	100µM FeSO <sub>4</sub>	<b>1.52+/-0.04</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>1.31+/-0.03</b>
	20µM Haem	<b>1.49+/-0.02</b>
	20µM Haem + 20µM ICL670A	<b>1.32+/-0.01</b>
	20µM ICL670A	0.92+/-0.04

**Table 4-8 Relative ferroportin expression in benign and malignant breast cells following exposure to iron and chelators**

Ferroportin expression was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression +/- 2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.1.3 Hepcidin

Hepcidin expression was significantly reduced in both cell populations following culture with iron or haem alone (all  $p < 0.008$ ). This down-regulation was eliminated by adding ICL670A to the culture medium in the case of iron-loading for both cell lines, and haem-loading for HB2. Reduced expression persisted in MDA-MB-231 cells cultured with haem, despite adding ICL670A. ICL670A alone stimulated up-regulation of hepcidin expression in the HB2 cells ( $p = 0.0005$ ) but had no significant effect on MDA-MB-231. See figure 4.9.



**Figure 4.13 Relative hepcidin expression in benign and malignant breast cells following exposure to iron and chelators**

Expression of hepcidin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

Cell Type	Culture Medium	Relative Heparidin Expression
<b>HB2</b>	Control	1+/-0.05
	100µM FeSO <sub>4</sub>	<b>0.62+/-0.03</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1+/-0.02
	20µM Haem	<b>0.74+/-0.01</b>
	20µM Haem + 20µM ICL670A	1.08+/-0.04
	20µM ICL670A	<b>1.35+/-0.05</b>
<b>MDA-MB-231</b>	Control	1+/-0.03
	100µM FeSO <sub>4</sub>	<b>0.53+/-0.01</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	0.96+/-0.02
	20µM Haem	<b>0.49+/-0.02</b>
	20µM Haem + 20µM ICL670A	<b>0.94+/-0.02</b>
	20µM ICL670A	1+/-0.04

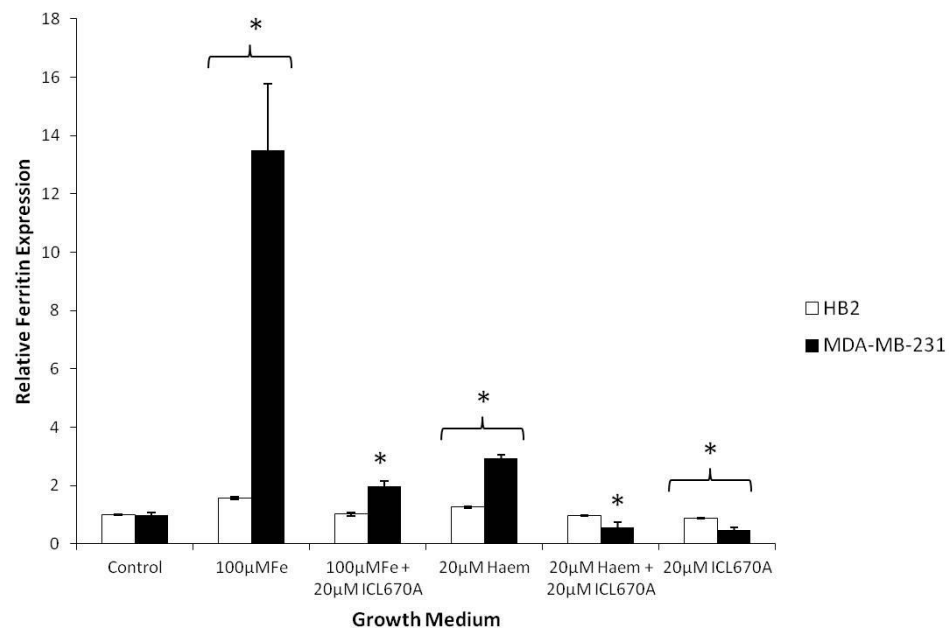
**Table 4-9 Relative hepcidin expression in benign and malignant breast cells following exposure to iron and chelators**

Hepcidin expression was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression +/- 2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.1.4 Iron Storage

Ferritin expression was significantly elevated in MDA-MB-231 cells following incubation with iron or haem in isolation, and with iron even in the presence of ICL670A (all  $p < 0.009$ ). Expression was significantly lower after incubation with haem in the presence of ICL670A and with ICL670A alone (both  $p < 0.03$ ).

Incubation with iron or haem alone stimulated up-regulation in ferritin expression in HB2 cells (both  $p < 0.0003$ ), although in both cases this was abrogated by the introduction of ICL670A. ICL670A alone again elicited a significant reduction in expressed ferritin levels ( $p = 0.002$ ).



**Figure 4.14 Relative expression of ferritin in benign and malignant cells following exposure to iron and chelators**

Protein expression of ferritin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).



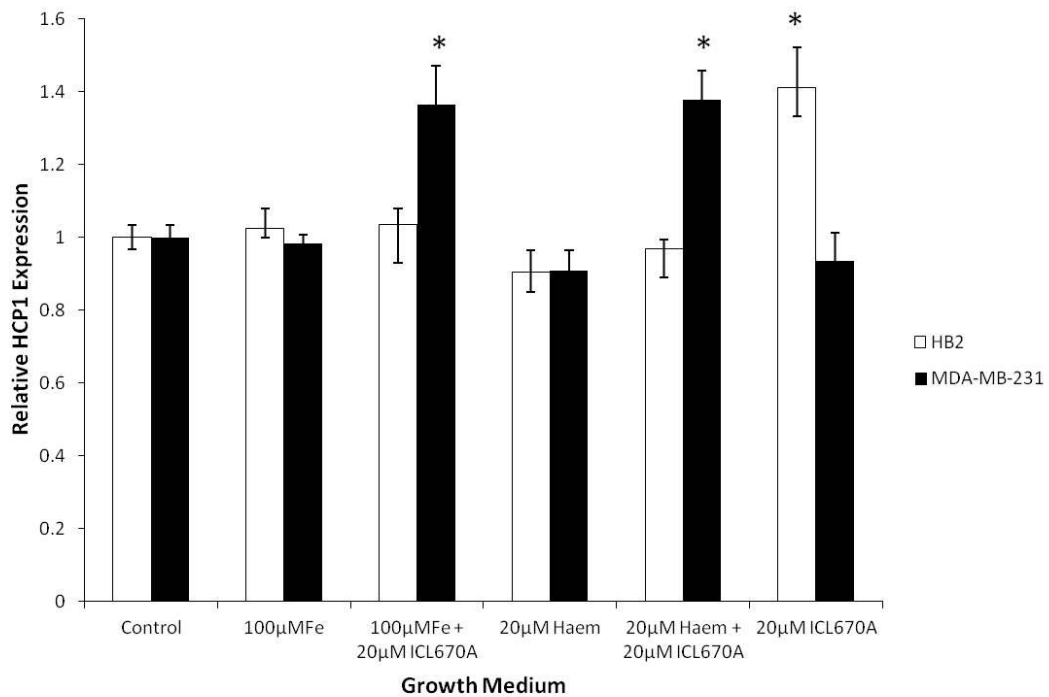
Cell Type	Culture Medium	Relative Ferritin Expression
<b>HB2</b>	Control	1+/-0.02
	100µM FeSO <sub>4</sub>	<b>1.58+/-0.05</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.03+/-0.06
	20µM Haem	<b>1.26+/-0.04</b>
	20µM Haem + 20µM ICL670A	0.97+/-0.01
	20µM ICL670A	<b>0.88+/-0.02</b>
<b>MDA-MB-231</b>	Control	1+/-0.07
	100µM FeSO <sub>4</sub>	<b>13.51+/-2.29</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>2+/-0.15</b>
	20µM Haem	<b>2.93+/-0.13</b>
	20µM Haem + 20µM ICL670A	<b>0.58+/-0.17</b>
	20µM ICL670A	<b>0.47+/-0.08</b>

**Table 4-10 Relative ferritin expression in benign and malignant breast cells following exposure to iron and chelators**

Ferritin expression was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.1.5 Haem Import Proteins

Expression of HCP1 was unchanged in both the benign and malignant lines following incubation with iron or haem alone. Malignant MDA-MB-231 cells significantly increased expression of HCP1 when the chelating agent was introduced alongside iron/haem (both  $p < 0.02$ ), although there was no response in the presence of a chelator in isolation. Conversely, HB2 cells showed no change in HCP1 expression when ICL670A was added to the iron/haem containing media, although levels increased significantly when ICL670A was introduced as a solitary reagent ( $p = 0.01$ ).



**Figure 4.15 Relative expression of HCP1 in benign and malignant cells following exposure to iron and chelators**

Protein expression of HCP1 was investigated after exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

Cell Type	Culture Medium	Relative HCP1 Expression
<b>HB2</b>	Control	1+/-0.03
	100µM FeSO <sub>4</sub>	1.02+/-0.06
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.03+/-0.04
	20µM Haem	0.91+/-0.06
	20µM Haem + 20µM ICL670A	0.97+/-0.03
	20µM ICL670A	<b>1.41+/-0.11</b>
<b>MDA-MB-231</b>	Control	1+/-0.03
	100µM FeSO <sub>4</sub>	0.98+/-0.02
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>1.36+/-0.11</b>
	20µM Haem	0.91+/-0.06
	20µM Haem + 20µM ICL670A	<b>1.38+/-0.08</b>
	20µM ICL670A	0.93+/-0.08

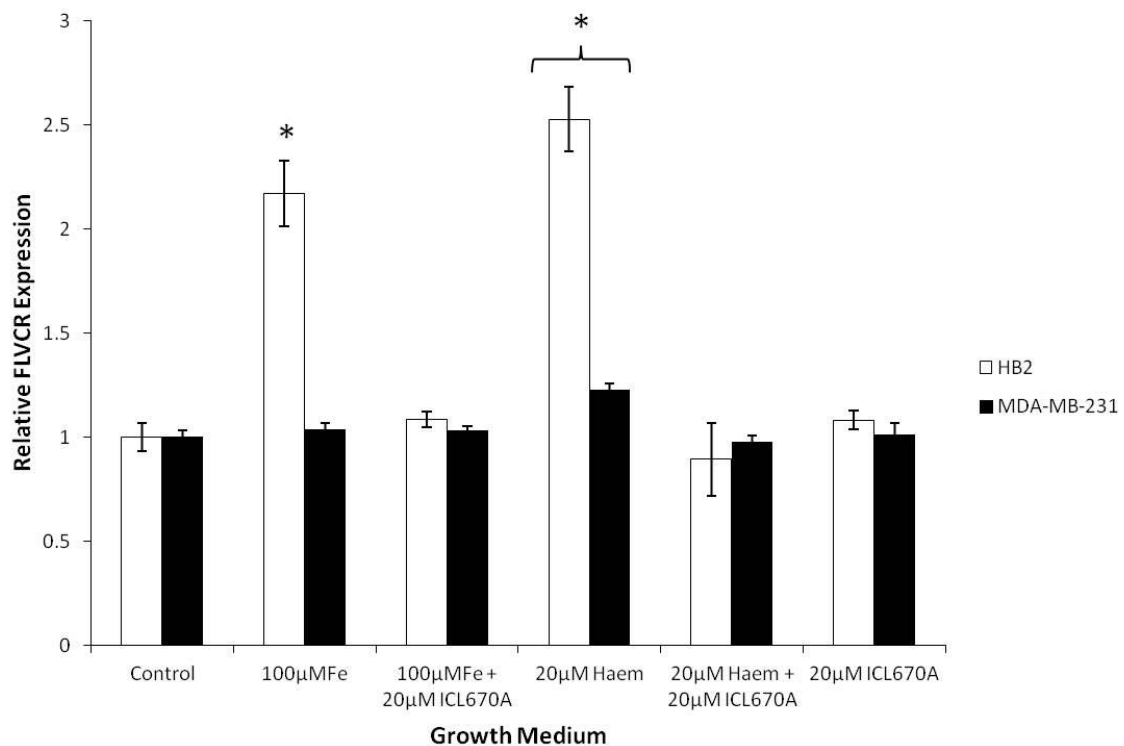
**Table 4-11 Relative HCP1 expression in benign and malignant breast cells following exposure to iron and chelators**

HCP1 expression was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.1.6 Haem Export Proteins

Iron and haem both induced benign HB2 cells to up-regulate expression of FLVCR when added to standard culture medium (both  $p < 0.002$ ). Expression returned to normal when ICL670A was also introduced. ICL670A alone had no effect.

Malignant MDA-MB-231 cells demonstrated increased FLVCR expression in response to the addition of haem ( $p = 0.0004$ ), but no other significant changes.



**Figure 4.16 Relative expression of FLVCR in benign and malignant cells following exposure to iron and chelators**

Protein expression of FLVCR was investigated after exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

Cell Type	Culture Medium	Relative FLVCR Expression
<b>HB2</b>	Control	1+/-0.07
	100µM FeSO <sub>4</sub>	<b>2.17+/-0.16</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.09+/-0.04
	20µM Haem	<b>2.53+/-0.16</b>
	20µM Haem + 20µM ICL670A	0.89+/-0.17
	20µM ICL670A	1.08+/-0.05
<b>MDA-MB-231</b>	Control	1+/-0.03
	100µM FeSO <sub>4</sub>	1.04+/-0.03
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.03+/-0.02
	20µM Haem	<b>1.23+/-0.03</b>
	20µM Haem + 20µM ICL670A	0.98+/-0.03
	20µM ICL670A	1.01+/-0.05

**Table 4-12 Relative FLVCR expression in benign and malignant breast cells following exposure to iron and chelators**

FLVCR expression was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative protein expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

### **4.3.2 Changes in Expression of Iron and Haem Transport Protein mRNA in Response to Iron and Chelators**

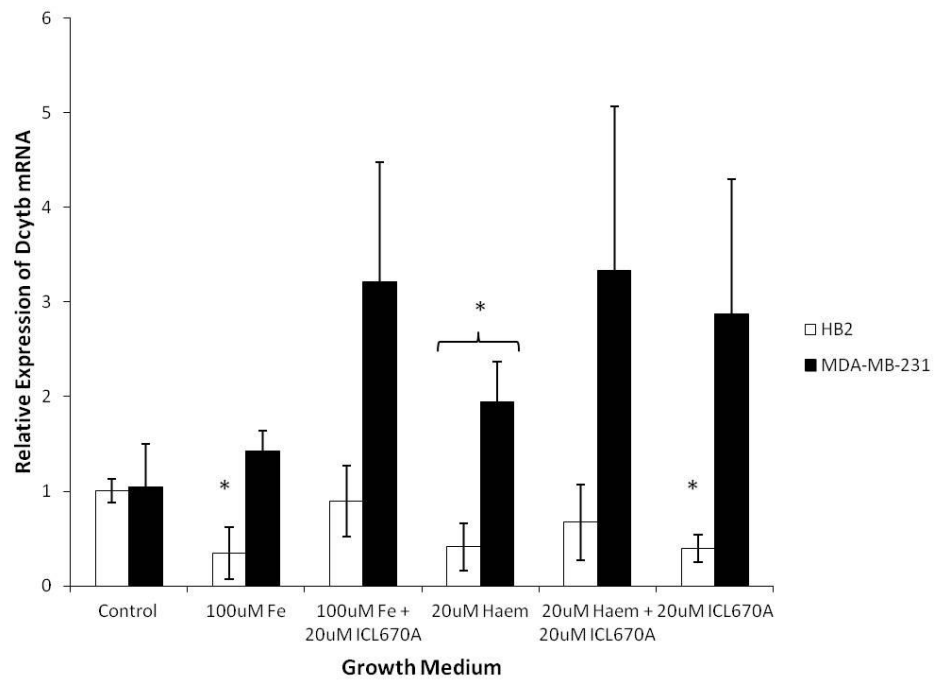
#### *4.3.2.1 Iron Import Proteins*

HB2 cells demonstrated a significant down-regulation of Dcytb mRNA in response to excess extracellular iron or haem (both  $p < 0.03$ ). The addition of ICL670A reversed the observed changes. ICL670A alone appeared to result in reduced expression ( $p = 0.003$ ). MDA-MB-231 cells exhibited no significant change when iron was added to culture medium. Inclusion of haem prompted significantly increased expression of Dcytb mRNA ( $p = 0.045$ ). ICL670A had no significant effect on Dcytb mRNA expression, whether alone or in combination with iron or haem.

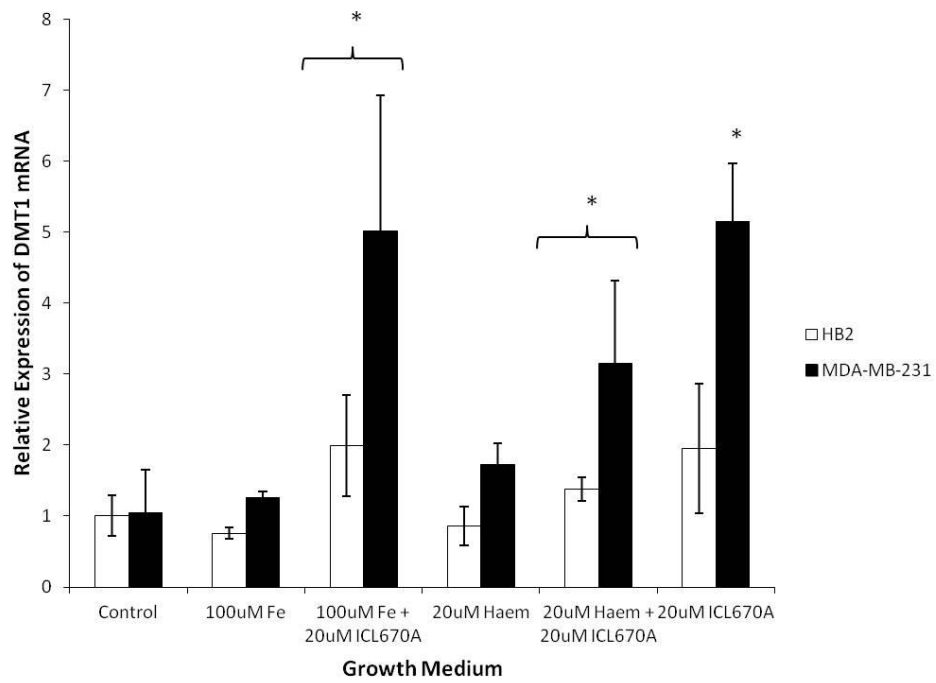
Neither HB2 nor MDA-MB-231 cells demonstrated a change in DMT1 mRNA levels after incubation with iron or haem in isolation, although both displayed significantly up-regulated expression when ICL670A was also present (all  $p < 0.04$ ). The malignant cells demonstrated similar behaviour following incubation with ICL670A alone ( $p = 0.0004$ ), although this pattern was not observed for HB2 cells.

TfR1 mRNA expression was significantly down-regulated by HB2 cells incubated with haem ( $p = 0.001$ ), but appeared unchanged with iron. Adding ICL670A returned expression to normal. ICL670A resulted in significantly up-regulated expression of TfR1 in the benign cells ( $p = 0.035$ ). MDA-MB-231 cells did not modify expression of TfR1 mRNA in response to either iron or haem, but significantly up-regulated expression in response to the presence of the chelator, whether in isolation or in conjunction with iron/haem (all  $p < 0.05$ ).

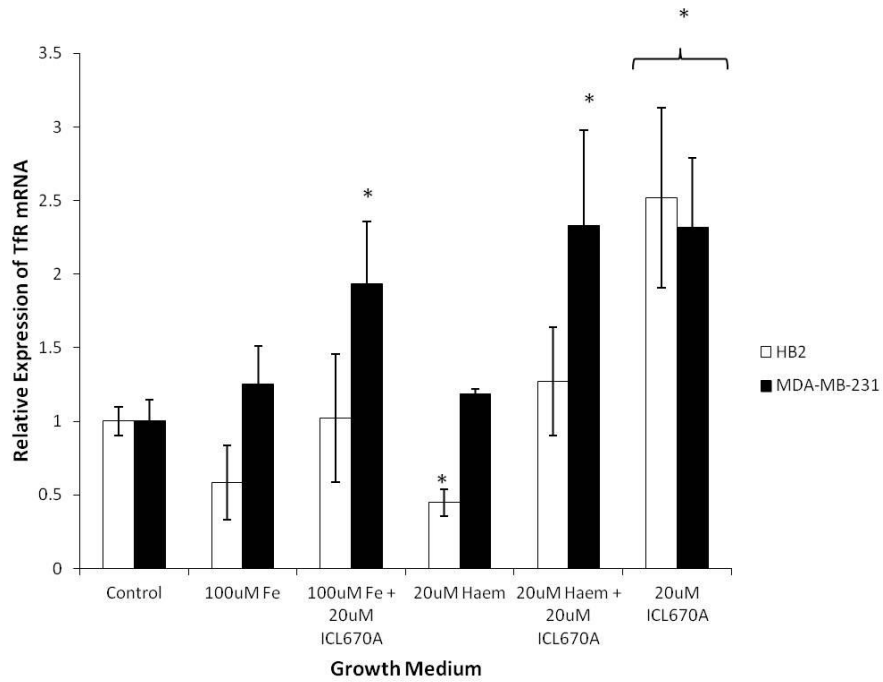
**A**



**B**



C



**Figure 4.17 Relative expression of iron import protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for Dcytb (A), DMT1 (B) and TfR1 (C) was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).



Cell Type	Experimental Medium	Relative Dcytb mRNA Expression	Relative DMT1 mRNA Expression	Relative TfR1 mRNA Expression
<b>HB2</b>	Control	1+/-0.12	1+/-0.28	1+/-0.1
	100µM FeSO <sub>4</sub>	<b>0.34+/-0.28</b>	0.76+/-0.08	0.59+/-0.25
	100µM FeSO <sub>4</sub> + 20µM ICL670A	0.89+/-0.37	<b>1.99+/-0.71</b>	1.02+/-0.43
	20µM Haem	<b>0.41+/-0.25</b>	0.85+/-0.27	<b>0.45+/-0.09</b>
	20µM Haem + 20µM ICL670A	0.67+/-0.4	<b>1.38+/-0.17</b>	1.27+/-0.37
	20µM ICL670A	<b>0.4+/-0.14</b>	1.95+/-0.91	<b>2.52+/-0.61</b>
<b>MDA-MB-231</b>	Control	1+/-0.45	1+/-0.61	1+/-0.14
	100µM FeSO <sub>4</sub>	1.42+/-0.21	1.26+/-0.09	1.25+/-0.26
	100µM FeSO <sub>4</sub> + 20µM ICL670A	3.21+/-1.27	<b>5.01+/-1.92</b>	<b>1.93+/-0.42</b>
	20µM Haem	<b>1.95+/-0.43</b>	1.73+/-0.3	1.19+/-0.03
	20µM Haem + 20µM ICL670A	3.33+/-1.74	<b>3.16+/-1.17</b>	<b>2.33+/-0.65</b>
	20µM ICL670A	2.87+/-1.42	<b>5.15+/-0.81</b>	<b>2.32+/-0.47</b>

**Table 4-13 Relative expression of iron import protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

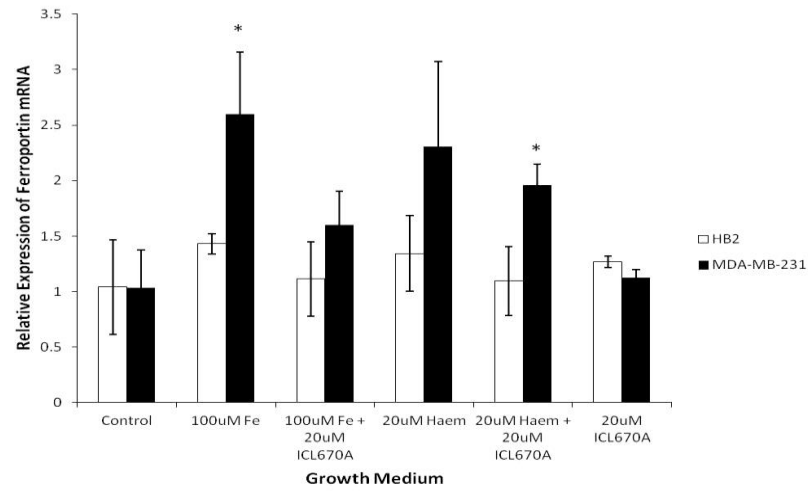
Expression of mRNA coding for Dcytb, DMT1 and TfR1 was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### *4.3.2.2 Iron Export Proteins*

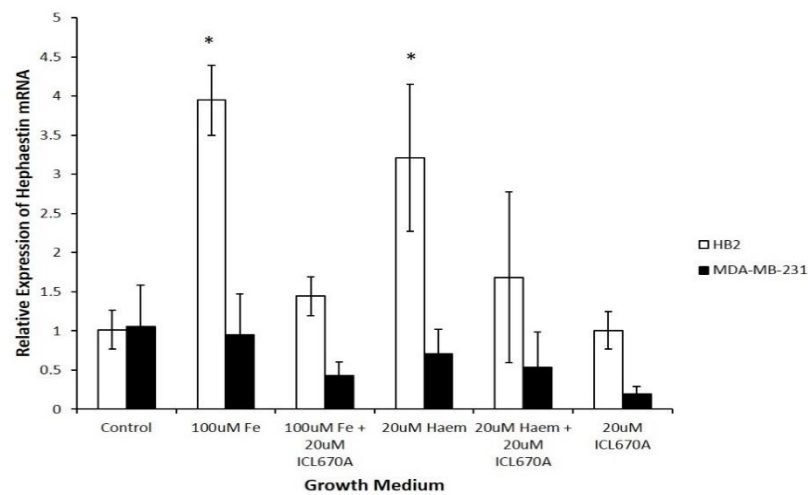
Expression of ferroportin mRNA was significantly up-regulated in MDA-MB-231 cells incubated with iron alone, and in the same cells following incubation with haem and ICL670A simultaneously (both  $p < 0.02$ ). Neither cell line demonstrated a significant response to any of the other experimental media.

Hephaestin mRNA was significantly over-expressed in benign HB2 cells incubated with either iron or haem alone (both  $p < 0.04$ ). Addition of a chelator abrogated this response, returning levels to normal. MDA-MB-231 cells did not modulate expression of hephaestin mRNA following incubation with any of the experimental media.

**A**



**B**



**Figure 4.18 Relative expression of iron export protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for ferroportin (**A**) and hephaestin (**B**) was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm$ 2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

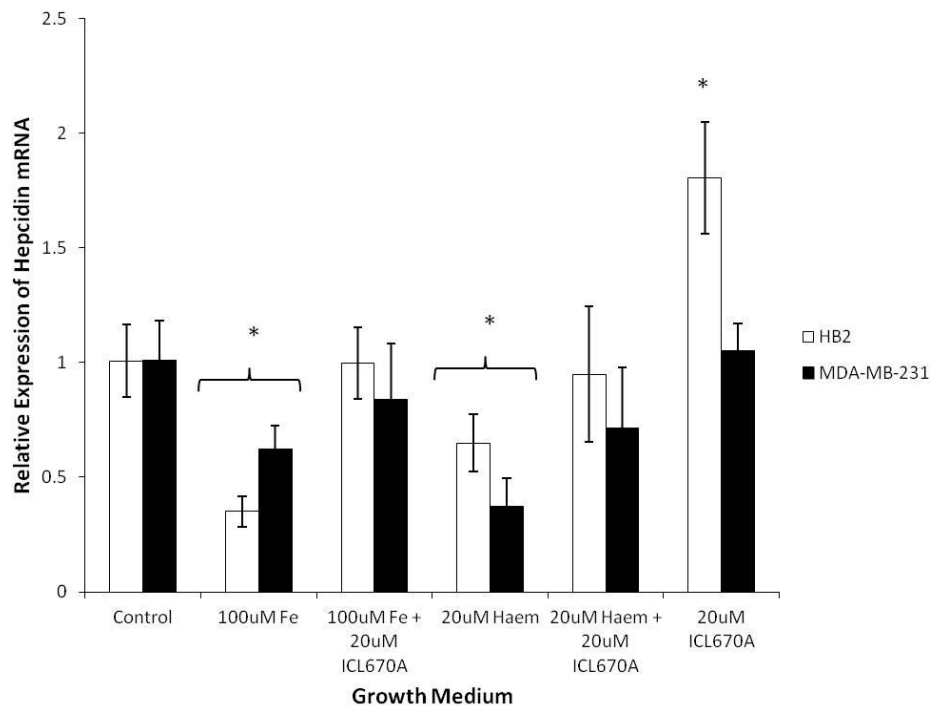
Cell Type	Experimental Medium	Relative ferroportin mRNA Expression	Relative hephaestin mRNA Expression
<b>HB2</b>	Control	1+/-0.43	1+/-0.25
	100µM FeSO <sub>4</sub>	1.43+/-0.09	<b>3.95+/-0.45</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.11+/-0.33	1.44+/-0.25
	20µM Haem	1.34+/-0.34	<b>3.21+/-0.94</b>
	20µM Haem + 20µM ICL670A	1.1+/-0.31	1.68+/-1.09
	20µM ICL670A	1.27+/-0.53	1.01+/-0.24
<b>MDA-MB-231</b>	Control	1+/-0.34	1+/-0.53
	100µM FeSO <sub>4</sub>	<b>2.6+/-0.59</b>	0.95+/-0.52
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.6+/-0.31	0.43+/-0.17
	20µM Haem	2.3+/-0.77	0.71+/-0.31
	20µM Haem + 20µM ICL670A	<b>1.96+/-0.19</b>	0.54+/-0.45
	20µM ICL670A	1.13+/-0.07	0.2+/-0.91

**Table 4-14 Relative expression of iron export protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for ferroportin and hephaestin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.2.3 Hepcidin

Both HB2 and MDA-MB-231 cells exhibited reduced expression of hepcidin mRNA after incubation with either iron or haem alone (all  $p < 0.03$ ). Addition of ICL670A reversed the change in both cases. HB2 cells also displayed over-expression of hepcidin mRNA following incubation with ICL670A alone ( $p = 0.008$ ).



**Figure 4.19 Relative expression of hepcidin mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for hepcidin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm 2$ SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

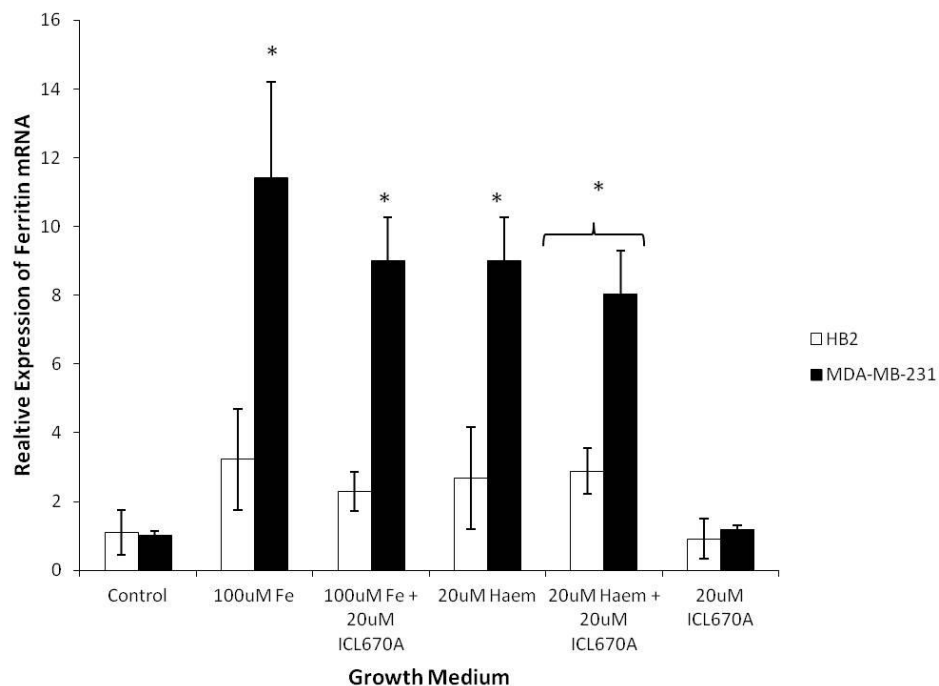
Cell Type	Culture Medium	Relative Heparidin mRNA Expression
<b>HB2</b>	Control	1+/-0.16
	100µM FeSO <sub>4</sub>	<b>0.35+/-0.07</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1+/-0.16
	20µM Haem	<b>0.65+/-0.13</b>
	20µM Haem + 20µM ICL670A	0.95+/-0.29
	20µM ICL670A	<b>1.81+/-0.24</b>
<b>MDA-MB-231</b>	Control	1+/-0.18
	100µM FeSO <sub>4</sub>	<b>0.62+/-0.1</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	0.84+/-0.25
	20µM Haem	<b>0.37+/-0.12</b>
	20µM Haem + 20µM ICL670A	0.71+/-0.27
	20µM ICL670A	1.05+/-0.12

**Table 4-15 Relative expression of hepcidin mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for hepcidin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### 4.3.2.4 Iron Storage

Ferritin mRNA levels were significantly increased in MDA-MB-231 cells exposed to iron or haem in isolation and in conjunction with ICL670A (all  $p < 0.02$ ). ICL670A alone had no effect. HB2 cells exhibited significantly increased ferritin mRNA expression after incubation with haem and ICL670A in combination ( $p = 0.018$ ), but were unaffected by any other combination of reagents.



**Figure 4.20 Relative expression of ferritin mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for ferritin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

Cell Type	Culture Medium	Relative Ferritin mRNA Expression
<b>HB2</b>	Control	1+/-0.66
	100µM FeSO <sub>4</sub>	3.22+/-1.47
	100µM FeSO <sub>4</sub> + 20µM ICL670A	2.3+/-0.56
	20µM Haem	2.68+/-1.58
	20µM Haem + 20µM ICL670A	<b>2.89+/-0.66</b>
	20µM ICL670A	0.92+/-0.59
<b>MDA-MB-231</b>	Control	1+/-0.13
	100µM FeSO <sub>4</sub>	<b>11.43+/-2.78</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>9.02+/-1.25</b>
	20µM Haem	<b>9+/-1.27</b>
	20µM Haem + 20µM ICL670A	<b>8.03+/-1.27</b>
	20µM ICL670A	1.19+/-0.13

**Table 4-16 Relative expression of ferritin mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for ferritin was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

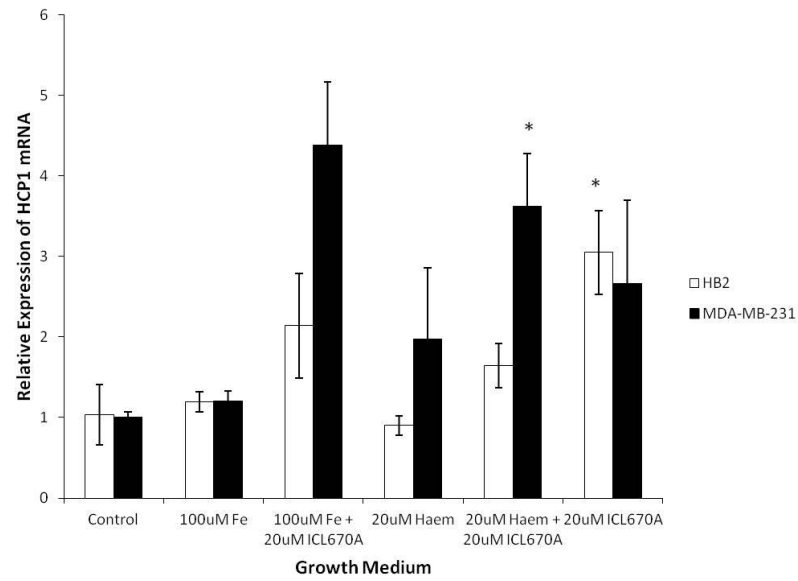


#### *4.3.2.5 Haem Import Proteins*

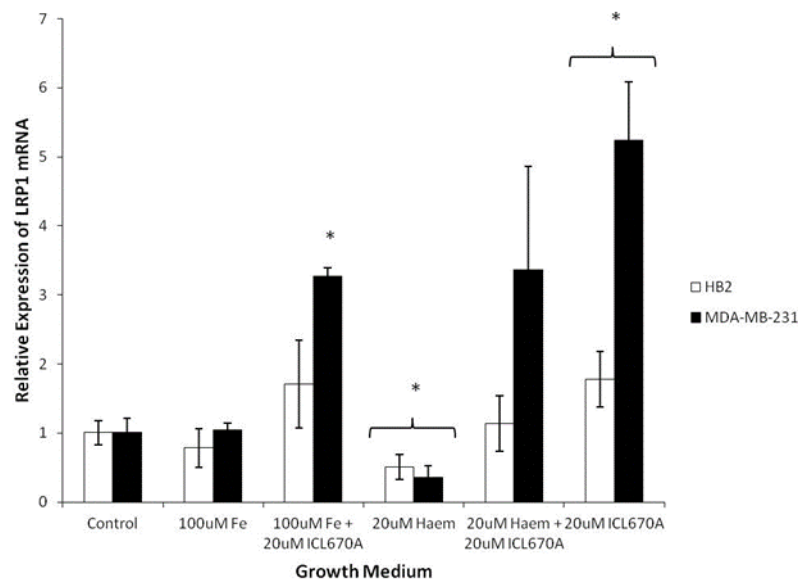
Neither HB2 cells nor MDA-MB-231 moderated expression of HCP1 mRNA in response to incubation with either iron or haem. Adding a chelator to iron/haem in the culture medium induced significant up-regulation in MDA-MB-231 cells (both  $p < 0.02$ ); expression was once again unaltered in HB2 cells. ICL670A in isolation induced significantly higher levels of HCP1 mRNA in HB2 cells ( $p = 0.005$ ) but not in the MDA-MB-231 line.

Incubation with iron alone did not affect LRP1 mRNA expression in either cell line. Adding ICL670A to the iron resulted in significantly higher levels of LRP1 mRNA in MDA-MB-231 cells ( $p = 0.0002$ ) but did not influence the HB2 line. Haem in isolation caused LRP1 mRNA expression to be significantly inhibited in both cell lines (both  $p < 0.02$ ); addition of ICL670A restored levels to normal in both cases. ICL670A alone stimulated significant up-regulation of LRP1 mRNA expression in both cell lines (both  $p < 0.05$ ).

**A**



**B**



**Figure 4.21 Relative expression of haem import protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for HCP1 (**A**) and LRP1 (**B**) was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

Cell Type	Experimental Medium	Relative HCP1 mRNA Expression	Relative LRP mRNA Expression
<b>HB2</b>	Control	1+/-0.38	1+/-0.17
	100µM FeSO <sub>4</sub>	1.19+/-0.13	0.78+/-0.28
	100µM FeSO <sub>4</sub> + 20µM ICL670A	2.14+/-0.65	1.71+/-0.63
	20µM Haem	0.9+/-0.12	<b>0.5+/-0.18</b>
	20µM Haem + 20µM ICL670A	1.64+/-0.27	1.13+/-0.4
	20µM ICL670A	<b>3.05+/-0.52</b>	<b>1.78+/-0.4</b>
<b>MDA-MB-231</b>	Control	1+/-0.07	1+/-0.2
	100µM FeSO <sub>4</sub>	1.2+/-0.13	1.05+/-0.09
	100µM FeSO <sub>4</sub> + 20µM ICL670A	<b>4.38+/-0.79</b>	<b>3.28+/-0.12</b>
	20µM Haem	1.97+/-0.89	<b>0.36+/-0.16</b>
	20µM Haem + 20µM ICL670A	<b>3.62+/-0.66</b>	3.37+/-1.5
	20µM ICL670A	2.66+/-1.04	<b>5.24+/-0.85</b>

**Table 4-17 Relative expression of HCP1 and LRP mRNA in benign and malignant breast cells following exposure to iron and chelators**

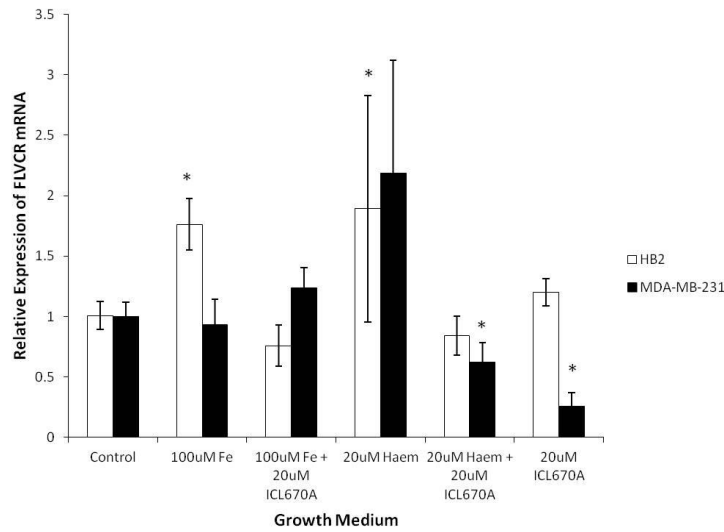
Expression of mRNA coding for HCP1 and LRP was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### *4.3.2.6 Haem Export Proteins*

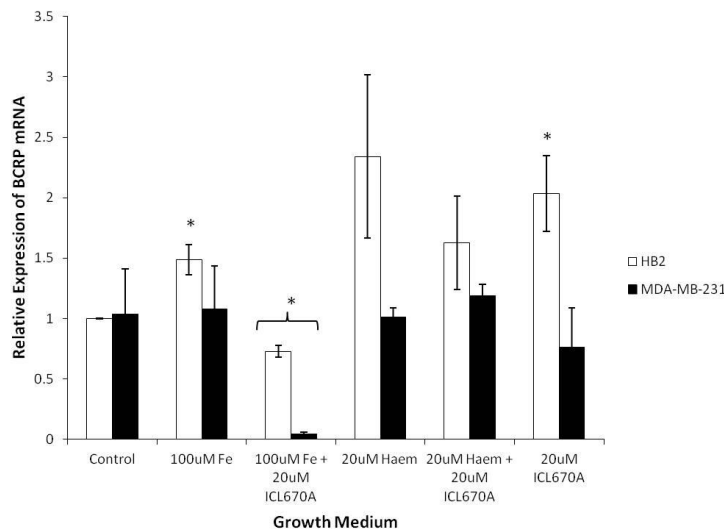
Expression of FLVCR mRNA was significantly inhibited in HB2 cells following incubation with either iron or haem (both  $p < 0.02$ ). Adding ICL670A to these culture media restored expression to normal levels. ICL670A alone had no effect on FLVCR mRNA expression in HB2 cells. MDA-MB-231 cells did not modulate their expression of FLVCR mRNA when exposed to either iron or haem, although adding ICL670A to the haem did induce significant down-regulation, as did ICL670A alone (both  $p < 0.02$ ).

BCRP mRNA expression was significantly increased in HB2 cells following incubation with iron ( $p = 0.016$ ), but not haem. Adding ICL670A to the iron led to significant down-regulation in expression ( $p = 0.008$ ). Incubation with ICL670A alone also appeared to significantly increase BCRP mRNA levels in the benign cells ( $p = 0.021$ ). MDA-MB-231 cells demonstrated a significant inhibition of BCRP mRNA levels after exposure to iron and ICL670A in combination ( $p = 0.034$ ). No other experimental media stimulated any change in expression.

**A**



**B**



**Figure 4.22 Relative expression of haem export protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for FLVCR (**A**) and BCRP (**B**) was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).

Cell Type	Experimental Medium	Relative FLVCR mRNA Expression	Relative BCRP mRNA Expression
<b>HB2</b>	Control	1+/-0.19	1+/-0.0
	100µM FeSO <sub>4</sub>	<b>1.76+/-0.32</b>	<b>1.49+/-0.13</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	0.76+/-0.13	<b>0.73+/-0.05</b>
	20µM Haem	<b>1.89+/-0.26</b>	2.34+/-0.67
	20µM Haem + 20µM ICL670A	0.84+/-0.12	1.62+/-0.39
	20µM ICL670A	1.2+/-0.15	<b>2.03+/-0.31</b>
<b>MDA-MB-231</b>	Control	1+/-0.16	1+/-0.38
	100µM FeSO <sub>4</sub>	0.93+/-0.21	1.08+/-0.35
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.24+/-0.17	<b>0.05+/-0.01</b>
	20µM Haem	2.18+/-0.94	1.01+/-0.08
	20µM Haem + 20µM ICL670A	<b>0.62+/-0.16</b>	1.19+/-0.09
	20µM ICL670A	<b>0.26+/-0.11</b>	0.76+/-0.33

**Table 4-18 Relative expression of FLVCR and BCRP mRNA in benign and malignant breast cells following exposure to iron and chelators**

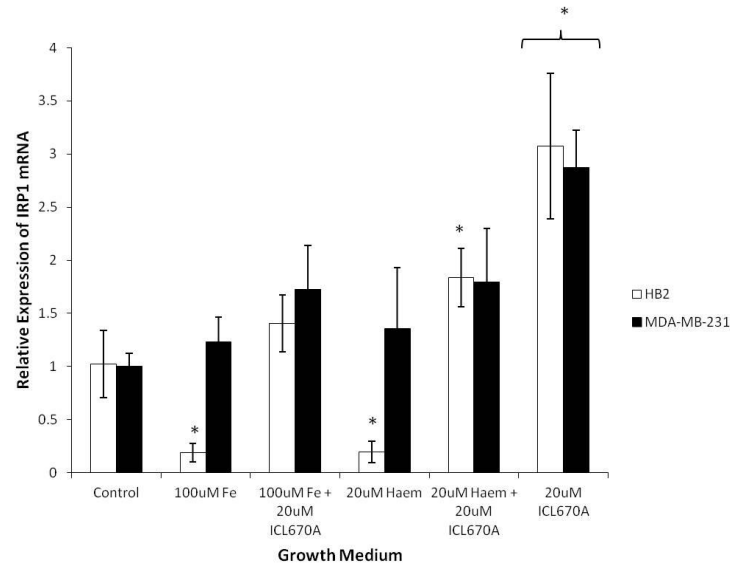
Expression of mRNA coding for FLVCR and BCRP was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

#### *4.3.2.7 Iron Regulatory Proteins*

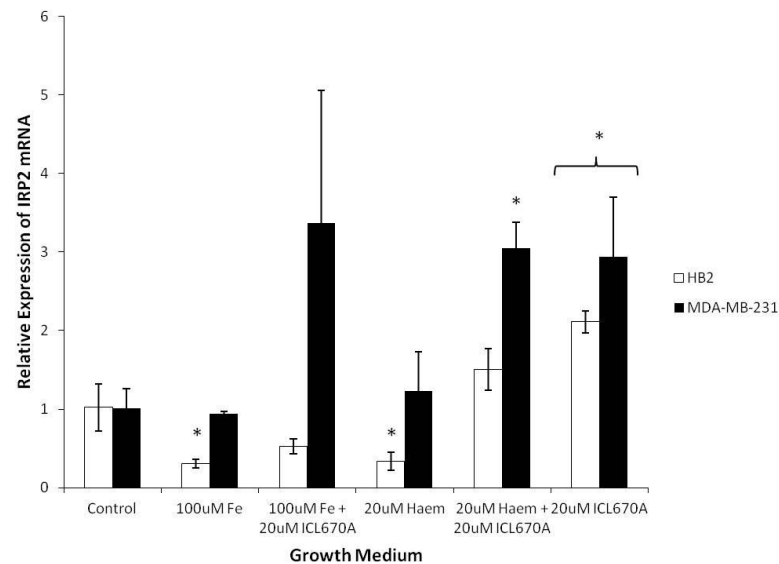
Both iron and haem significantly inhibited expression of IRP1 mRNA in HB2 cells (both  $p < 0.03$ ). ICL670A significantly increased IRP1 mRNA expression in HB2 cells with and without haem (both  $p < 0.02$ ). ICL670A in isolation led to significant up-regulation of IRP1 mRNA in MDA-MB-231 cells ( $p = 0.005$ ).

IRP2 expression was also significantly repressed in HB2 cells incubated with either iron or haem (both  $p < 0.04$ ). Haem and ICL670A in co-culture significantly increased IRP2 mRNA expression in MDA-MB-231 cells ( $p = 0.0008$ ). ICL670A alone significantly increased IRP2 mRNA expression in both HB2 and MDA-MB-231 cells (both  $p < 0.03$ ).

**A**



**B**



**Figure 4.23 Relative expression of iron regulatory protein mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for IRP1 (A) and IRP2 (B) was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression  $\pm$  2SEM. \* denotes statistical significance relative to control ( $p < 0.05$ , student t-test).



Cell Type	Experimental Medium	Relative IRP1 mRNA Expression	Relative IRP2 mRNA Expression
<b>HB2</b>	Control	1+/-0.32	1+/-0.3
	100µM FeSO <sub>4</sub>	<b>0.19+/-0.09</b>	<b>0.31+/-0.05</b>
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.41+/-0.27	0.53+/-0.1
	20µM Haem	<b>0.19+/-0.1</b>	<b>0.34+/-0.11</b>
	20µM Haem + 20µM ICL670A	<b>1.84+/-0.27</b>	1.51+/-0.27
	20µM ICL670A	<b>3.07+/-0.68</b>	<b>2.11+/-0.14</b>
<b>MDA-MB-231</b>	Control	1+/-0.12	1+/-0.24
	100µM FeSO <sub>4</sub>	1.23+/-0.24	0.94+/-0.03
	100µM FeSO <sub>4</sub> + 20µM ICL670A	1.73+/-0.41	3.37+/-1.69
	20µM Haem	1.36+/-0.57	1.24+/-0.49
	20µM Haem + 20µM ICL670A	1.79+/-0.51	<b>3.05+/-0.33</b>
	20µM ICL670A	<b>2.88+/-0.35</b>	<b>2.94+/-0.76</b>

**Table 4-19 Relative expression of IRP1 and IRP2 mRNA in benign and malignant breast cells following exposure to iron and chelators**

Expression of mRNA coding for IRP1 and IRP2 was investigated following exposure of benign HB2 and malignant MDA-MB-231 mammary cells to culture media containing iron or haem with or without ICL670A, and ICL670A alone. Results are expressed as mean relative mRNA expression +/-2SEM. Statistically significant results (p<0.05) are highlighted in bold.

			100µM FeSO4	100µM FeSO4 + 20µM ICL670A	20µM Haem	20µM Haem + 20µM ICL670A	20µM ICL670A
<b>HB2</b>	Dcytb	Protein	↓	↓	↓	↓	↓
		mRNA	↓	→	↓	→	↓
	DMT1	Protein	→	↑	→	→	→
		mRNA	→	↑	→	↑	→
	TfR1	Protein	↓	→	↓	→	↑
		mRNA	→	→	↓	→	↑
<b>MDA-MB-231</b>	Dcytb	Protein	→	↓	→	↓	↓
		mRNA	→	→	↑	→	→
	DMT1	Protein	→	↑	→	↑	↑
		mRNA	→	↑	→	↑	↑
	TfR1	Protein	→	↑	→	↑	↑
		mRNA	→	↑	→	↑	↑

**Table 4-20 Summary of changes in expression of the iron import proteins and their mRNA in response to iron, haem and ICL670A**

Benign HB2 and malignant MDA-MB-231 cells were incubated with iron, haem and ICL670A alone and in combination. Levels of the Dcytb, DMT1 and TfR1 and their mRNA were then assessed. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→).

			100μM FeSO4	100μM FeSO4 + 20μM ICL670A	20μM Haem	20μM Haem + 20μM ICL670A	20μM ICL670A
<b>HB2</b>	FPN	Protein	↑	↑	↑	↑	→
		mRNA	→	→	→	→	→
	Heph	Protein	-	-	-	-	-
		mRNA	↑	→	↑	→	→
	Hepcidin	Protein	↓	→	↓	→	↑
		mRNA	↓	→	↓	→	↑
<b>MDA-MB-231</b>	FPN	Protein	↑	↑	↑	↑	→
		mRNA	↑	→	→	↑	→
	Heph	Protein	-	-	-	-	-
		mRNA	→	→	→	→	→
	Hepcidin	Protein	↓	→	↓	↓	→
		mRNA	↓	→	↓	→	→

**Table 4-21 Summary of changes in expression of hepcidin and the iron export proteins and their mRNA in response to iron, haem and ICL670A**

Benign HB2 and malignant MDA-MB-231 cells were incubated with iron, haem and ICL670A alone and in combination. Levels of ferroportin and hepcidin were then assessed. Levels of hephaestin were undetectable on Western blotting with the available antibodies. mRNA for all 3 proteins was assayed. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→).

		100μM FeSO4	100μM FeSO4 + 20μM ICL670A	20μM Haem	20μM Haem + 20μM ICL670A	20μM ICL670A
<b>HB2</b>	Protein	↑	→	↑	→	↓
	mRNA	→	→	→	↑	→
<b>MDA-MB-231</b>	Protein	↑	↑	↑	↓	↓
	mRNA	↑	↑	↑	↑	→

**Table 4-22 Summary of changes in expression of ferritin and its mRNA in response to iron, haem and ICL670A**

Benign HB2 and malignant MDA-MB-231 cells were incubated with iron, haem and ICL670A alone and in combination. Levels of ferritin and its mRNA were then assessed. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→).

			100µM FeSO4	100µM FeSO4 + 20µM ICL670A	20µM Haem	20µM Haem + 20µM ICL670A	20µM ICL670A
<b>HB2</b>	HCP1	Protein	→	→	→	→	↑
		mRNA	→	→	→	→	↑
	LRP1	Protein	-	-	-	-	-
		mRNA	→	→	↓	→	↑
<b>MDA-MB-231</b>	HCP1	Protein	→	↑	→	↑	→
		mRNA	→	↑	→	↑	→
	LRP1	Protein	-	-	-	-	-
		mRNA	→	↑	↓	→	↑

**Table 4-23 Summary of changes in expression of the haem import proteins and their mRNA in response to iron, haem and ICL670A**

Benign HB2 and malignant MDA-MB-231 cells were incubated with iron, haem and ICL670A alone and in combination. Levels of HCP1 were then assessed. LRP1 levels were undetectable on Western blotting with the antibodies available to us. mRNA for both proteins was assayed. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→).

			100µM FeSO <sub>4</sub>	100µM FeSO <sub>4</sub> + 20µM ICL670A	20µM Haem	20µM Haem + 20µM ICL670A	20µM ICL670A
<b>HB2</b>	FLVCR	Protein	↑	→	↑	→	→
		mRNA	↑	→	↑	→	→
	BCRP	Protein	-	-	-	-	-
		mRNA	↑	↓	→	→	↑
<b>MDA-MB-231</b>	FLVCR	Protein	→	→	↑	→	→
		mRNA	→	→	→	↓	↓
	BCRP	Protein	-	-	-	-	-
		mRNA	→	↓	→	→	→

**Figure 4.24 Summary of changes in expression of the haem export proteins and their mRNA in response to iron, haem and ICL670A**

Benign HB2 and malignant MDA-MB-231 cells were incubated with iron, haem and ICL670A alone and in combination. Levels of FLVCR were then assessed. BCRP levels were undetectable on Western blotting with the antibodies available to us. mRNA for both proteins was assayed. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→).

		100μM FeSO4	100μM FeSO4 + 20μM ICL670A	20μM Haem	20μM Haem + 20μM ICL670A	20μM ICL670A
<b>HB2</b>	IRP1 mRNA	↓	→	↓	↑	↑
	IRP2 mRNA	↓	→	↓	→	↑
<b>MDA-MB-231</b>	IRP1 mRNA	→	→	→	→	↑
	IRP2 mRNA	→	→	→	↑	↑

**Figure 4.25 Summary of changes in expression of the iron regulatory protein mRNA in response to iron, haem and ICL670A**

Benign HB2 and malignant MDA-MB-231 cells were incubated with iron, haem and ICL670A alone and in combination. Western blotting did not reveal any detectable IRP1 or IRP2 using the antibodies available to us. mRNA for both proteins was assayed. The arrows denote significant up- (↑) or down-regulation (↓), or no effect (→).

## 4.4 Summary and Discussion

Iron and haem have been implicated as potential carcinogens in the development of a number of epithelial cancers, including breast.

Utilising one benign and two malignant mammary cell lines, it was possible to explore differences in their respective ability to sequester iron and haem, and to investigate whether differential absorption produced quantifiable changes in cell phenotype resulting in more aggressive patterns of behaviour.

Initial incubation with  $\text{FeSO}_4$  and haem at various concentrations demonstrated that both benign and malignant cells were able to import non-haem iron from extracellular solution. However, only the malignant cell lines MCF7 and MDA-MB-231 exhibited increased concentrations of iron following incubation with haem. Further, the more avid importer of haem iron was the more aggressive MDA-MB-231 line. These results validate the hypothesis that more aggressive breast cancer phenotypes display greater capability to import iron, including haem. This is a novel finding.

Measuring cell viability demonstrated positive effects due to  $\text{FeSO}_4$  on both benign HB2 cells and the two malignant lines. This is not surprising, given that iron is a vital component of many cellular processes. Haem had no positive influence on HB2 viability and was in fact detrimental at higher concentrations. The more indolent of the malignant lines, MCF7, responded positively to low concentrations of haem, but also suffered a loss of viability as concentration increased. The aggressive MDA-MB-231 cells displayed increased viability across the range of haem concentrations with which they were incubated. This may be because their higher turnover allows malignant cells to utilise excess iron more quickly than benign cells, generating less of a labile iron pool and therefore fewer of the harmful free radicals and reactive oxygen species which are detrimental to cell health.



Although these data are suggestive that increased intracellular iron could be responsible for conferring a malignant phenotype, the trends observed could still conceivably be explained through increased iron utilisation by aggressive cells undergoing frequent cycling.

Similarly, all 3 cell lines exhibited augmented proliferation when incubated with  $\text{FeSO}_4$ , as expected given that proliferation is essentially ubiquitous to all cells and is dependent on ample supplies of iron to allow proper functioning of ribonucleotide reductase and the cyclins. Again, haem significantly up-regulated proliferation in the malignant cell lines, although interestingly it was the less aggressive MCF7 line that seemed to benefit the most. It is harder to explain why haem appeared to have a positive effect on proliferation of benign HB2 cells. Given that HB2 cells were earlier shown to be incapable of importing haem iron, it is difficult to see how this can be rationalised unless HB2 cells are in fact able to take up haem from solution, but lack the ability to liberate iron from haem molecules such that it can be detected by a ferrozine assay.

The ability of cells seeded individually to form colonies in agar gel can be extrapolated to estimate their potential to metastasize *in vivo*. Unlike proliferation, the ability to generate colonies from single cells is not a feature of normal cell behaviour, so it is therefore unsurprising that iron and haem had no effect on HB2 cells. Conversely, MCF7 and MDA-MB-231 cells were both stimulated to up-regulate colony formation in response to the presence of iron or haem in the agar. MCF7 cells exhibited a significant rise in number of colonies formed with the introduction of either additive, although in neither case did average colony size increase. MDA-MB-231 cells were driven to increase both the size and number of colonies forming when iron was present and produced larger colonies in the presence of haem. Studying anchorage-independent growth provided the first evidence of truly divergent responses to supplemental iron between benign and malignant mammary cells and produced

the first compelling proof that iron can augment malignant behaviour. It was again instructive to note that the more aggressive cancer cells were the most responsive to increasing iron levels. Investigating the mechanism behind the positive effects of iron and haem on the anchorage-independent growth of breast cancer cells fell outside the scope of this study. It is possible that this effect is mediated via the cytoplasmic protein NDRG1. This protein is involved in stress responses and cell differentiation and is a known suppressor of tumour metastasis<sup>434,435</sup>. NDRG1 expression is amplified when cellular iron levels fall<sup>427</sup>. It may be that increased intra-cellular iron up-regulates anchorage-independent growth via an inhibitory effect on NDRG1 and that the elevated iron levels induced in these experiments had an anti-NDRG1 effect allowing increased colony formation.

Migratory capacity is a further phenotypic trait that is more pronounced in malignant cells. Although there was a degree of closure of wounds formed in HB2 monolayers, this was inhibited by iron and haem. Migration of MCF7 and MDA-MB-231 cells was significantly up-regulated by both iron and haem, reinforcing the concept of iron-driven augmentation of aggressive behaviour in malignant cells.

Further evidence emerged from invasion assays demonstrating HB2 cells to be minimally invasive, and displaying no up-regulation in invasive capacity following introduction of iron or haem to transwell inserts. MCF7 and MDA-MB-231 cells incubated with iron or haem exhibited significantly greater invasion through a matrigel membrane than control cells. Previous studies have suggested that a major factor permitting increased motility and invasiveness of malignant cells in epithelial neoplasms is repression of E-cadherin, an adherens junction protein. Lack of function of E-cadherin reduces the integrity of intercellular junctions allowing malignant cells to break free and disperse<sup>428</sup>. It has been shown that iron has an inhibitory effect on E-cadherin function in both hepatocellular and colorectal

carcinoma<sup>319</sup>. Loss of E-cadherin is also strongly implicated in the progression of breast cancer<sup>429</sup> and although the role of iron in this context has never been investigated it is possible that increased iron levels may play a similar inhibitory role to that observed in other epithelial malignancies.

In summary, iron-loading studies proved that malignant mammary cells are capable of importing and utilising iron in both its ferrous and haem forms. Subsequently, iron and haem were shown to be capable of up-regulating an array of malignant traits in breast cancer cells. Although HB2 cells can utilise a degree of supplemental iron to increase viability and proliferation, this is easily explained by the presence of a more plentiful supply of an essential cell nutrient. HB2 cells did not exhibit any increase in aggressive behavioural traits in response to iron or haem. These findings support the hypothesis that the dysregulation in iron and haem transport proteins demonstrated previously in breast cancer specimens could be acting to mediate a carcinogenic process.

The use of iron chelators as a potential adjunct in management of breast cancer is a novel idea which is gaining traction in the breast cancer community. If the concepts outlined above are accurate, it would be logical to assume that depriving breast cancer cells of iron would suppress their aggressive behaviour. Whether the alterations in iron trafficking and cell phenotype in malignant lines conferred by increased availability of iron betray a previously unsuspected carcinogenic pathway, or merely reflect increased demand for iron due to behavioural changes in cancer, malignant cells still depend on increased iron levels for survival. It was therefore instructive to observe the effect of ICL670A on cell phenotype. Iron chelation inhibited the previously observed up-regulation in viability and proliferation induced in MCF7 and MDA-MB-231 cells by iron and haem. There were also negative effects on these phenotypic traits when ICL670A was added to growth medium in the absence of stimulant

additives, implying a detrimental effect on the normal functioning of malignant breast cells. Iron chelation was shown to inhibit proliferation in benign HB2 cells relative to control, indicating that some of the negative effects on cell health and functioning are not confined to malignant populations, and are therefore likely due to insufficient iron being available to support normal cell metabolism.

Adding ICL670A to agar gels abrogated the positive effects of iron and haem on anchorage-independent growth of MCF7 and MDA-MB-231 cells, and significantly inhibited colony formation in un-stimulated cells relative to control. The consequences of adding ICL670A to wound-healing assays were less definitive. Although rates of migration in malignant lines treated with the chelator fell relative to control or the relevant stimulant medium, these changes were not overt and did not achieve statistical significance. In contrast, ICL670A had a marked inhibitory effect on the invasive capacity of malignant breast cells, both reversing the positive influence of iron and haem on invasion and significantly inhibiting invasion in untreated cells relative to control.

Iron chelating agents have already been demonstrated to have anti-tumour effects against oesophageal carcinoma<sup>430</sup> and melanoma<sup>426</sup>, among others. A recent study demonstrated induction of apoptosis in MCF-7 and MDA-MB-231 cells in response to administration of the chelator desferrioxamine, as well as an increased susceptibility to chemotherapeutic agents<sup>431</sup>. Our results, using a wider range of phenotype assays than previous groups have reported, would appear to confirm the anti-tumour effect of iron chelators on breast cancer cells.

Studying the effects of iron and haem loading on benign and malignant mammary cells in tissue culture provided further evidence that expression of the iron and haem transport proteins and their mRNA is inappropriately controlled in breast cancer.

Expression of Dcytb and TfR1 and their mRNA decreased appropriately when HB2 cells were incubated with iron and haem, whereas malignant MDA-MB-231 cells maintained baseline expression. Adding ICL670A led to increased production of TfR1 protein and mRNA in MDA-MB-231 control cells, and those incubated with iron or haem, whereas only control HB2 cells demonstrated the same response. Expression of DMT1 protein and mRNA was unchanged with iron or haem loading in either population although addition of a chelator appeared to stimulate production to a far greater extent in malignant cells. Ferroportin expression increased in both cells lines in response to iron or haem loading, as would be expected. This was accompanied by a reduction in hepcidin expression, which would act to preserve ferroportin levels. Thus, although both cell lines up-regulated their iron export machinery in response to excess extra-cellular iron or haem, only benign cells exhibited a synergistic down-regulation of iron import. Intra-cellular iron levels in benign cells would therefore be expected to remain close to normal, whereas those in the malignant line would be likely to climb. This was demonstrated when ferritin levels were seen to rise to a far greater extent in malignant cells in response to loading with iron or haem. At the mRNA level, iron and haem loading had no effect on HB2 cells, whereas MDA-MB-231 cells increased ferritin mRNA production markedly to sequester as much of the available iron as possible, rather than exporting it. Interestingly, the addition of ICL670A to control medium led to a greater reduction in ferritin expression in MDA-MB-231 than in HB2 cells, potentially demonstrating increased susceptibility to iron chelation in malignant populations.

Although there was no significant change in expression of haem import proteins in either cell line in response to iron or haem loading alone, the addition of a chelator to malignant cells already exposed to excess iron or haem significantly up-regulated HCP1 protein and mRNA production. This effect was not duplicated in the HB2 cells, although these did express both

HCP1 protein and mRNA in response to ICL670A alone, which MDA-MB-231 cells did not. LRP1 mRNA production was appropriately down-regulated in both populations following incubation with haem and significantly up-regulated in both in the presence of the chelator. Expression of the haem export protein FLVCR was significantly elevated in HB2 cells following incubation with either reagent, and in MDA-MB-231 cells in response to excess haem only. BCRP mRNA levels increased significantly in HB2 cells after iron or haem loading, but were unchanged in MDA-MB-231 cells. The effects of the observed changes in haem transport protein expression in response to increased extra-cellular iron or haem are therefore similar to the iron transport chain: haem importer expression is apparently unchanged in either population, but haem export is up-regulated to a greater extent in benign cells. The shift in balance between import and export would allow benign cells to expel excess iron, whereas the malignant cells retained more of their normal haem transporter profile in an iron-rich environment, which could be permissive for the accumulation of intra-cellular iron. The observed down-regulation of IRP1 and IRP2 mRNA expression in HB2 cells in response to iron and haem loading reflects an appropriate response aimed at minimising import of further iron. There was no such reaction in the malignant cells, with normal expression of IRP1 and IRP2 mRNA maintained in the face of increasing levels of  $\text{FeSO}_4$  and haem. This matches the unchanged expression of DMT1 and TfR1 in iron-rich conditions and implicates dysregulation of iron regulatory protein expression as a potential underlying factor in the development of an iron-hungry phenotype leading to a carcinogenic environment. Although multiple studies have previously demonstrated a clear relationship between dysregulation of non-haem iron transporters and breast cancer, most have been limited to reporting an up-regulation in iron import along with inhibition of export leading to an increase in intra-cellular iron levels. Our results report the responses of cultured cells to dynamic

changes in the availability of iron and demonstrate that breast cancer cells will attempt to sequester iron even when there is a huge surplus and intra-cellular reserves are plentiful. Although this could perhaps have been assumed from historic studies, it has not been formally reported until now.

## CHAPTER 5: CONCLUSION

There is mounting evidence that dysregulation in the way iron is handled by tissues may play a part in carcinogenesis. The majority of earlier research was performed on gastro-intestinal cancer but more recent work has demonstrated that breast carcinogenesis may also be due at least in part to similar changes in iron processing.

Most of the available evidence focusses on the proteins responsible for transport of ferric and ferrous iron, with no investigation into haem metabolism.

It has previously been demonstrated that levels of the iron import proteins TfR and DMT1, and the iron storage protein ferritin, are elevated in breast cancer relative to normal tissue. This is accompanied by down-regulation of the exporter ferroportin. This would clearly lead to increased intra-cellular iron and it has also been shown that higher levels of iron correspond to a more malignant phenotype in breast cancer.

Iron chelation has been investigated as a possible therapeutic modality in a number of different cancers, and recent preliminary research has indicated a potential role in breast cancer treatment.

This thesis aimed to confirm previously reported changes in the expression of non-haem iron transport proteins in breast cancer, as well as to examine the expression profiles of other transporters of haem and non-haem iron. We also set out to examine the effects of increasing intra-cellular iron levels on the behaviour of both benign and malignant breast cell lines in tissue culture, and how their expression of iron and haem transporters was altered in response to the availability of iron. Finally, we sought to further clarify the utility of iron chelators in breast cancer treatment through investigation of how the behaviour of both benign and malignant cells changed in response to chelation of extra-cellular iron.



We began by demonstrating that there is a clear difference in the expression profiles of both iron and haem transporters in breast carcinoma relative to matched normal tissue. We confirmed the up-regulation of the importers DMT1 and TfR1, increased ferritin expression and decreased expression of ferroportin that has previously been described (albeit in non-matched samples), as well as replicating previous results demonstrating increased hepcidin expression. However we also report the novel finding that Dcytb appears to be over-expressed in breast cancer, and that haem export is down-graded with significantly lower levels of expression of FLVCR and BCRP. We also demonstrated the previously un-reported finding of stromal staining for the iron import proteins Dcytb, DMT1 and TfR1 around deposits of breast cancer which may represent some form of peri-tumoural field change in the same way that certain cancers generate marked inflammation in the surrounding tissues.

Tissue culture experiments revealed that although benign and malignant cell lines are both capable of importing non-haem iron (as would be expected given its crucial role in metabolism), only malignant cells could import haem. Iron was shown to have positive effects on viability and proliferation in all cell populations, which was also anticipated, but only had a positive effect on what could be deemed “aggressive” phenotypic traits (colony forming, migration, invasion etc) in malignant cell lines.

Iron chelation was found to have significant anti-cancer effects when added to incubation media for a range of phenotypic assays, which tallies with previous results in other cancer populations. This also supports published data on the effect of iron chelation on breast cancer cells in vitro, although previous in vitro studies on breast cancer have tended to focus solely on the anti-proliferative effects of chelation. Our results showing anti-migratory and anti-invasive effects, as well as inhibition of colony formation, demonstrate the multi-factorial efficacy of iron chelation against cancer cell behaviour.

Investigation of iron and haem transporter expression in response to increasing exogenous iron and haem demonstrated that benign cells change their expression profiles to maintain normal levels of intra-cellular iron (via up-regulation of export and inhibiting import), whereas malignant cells will continue to behave in an “iron-hungry” manner, with normal expression of the import proteins, regardless of the availability of iron or intra-cellular iron concentration. Although malignant cells do require slightly more iron due to higher turnover and cell cycling, it is unlikely that this explains the significant differences in protein expression. It is more likely that protein expression is inappropriately dysregulated, and that the resulting increase in labile iron levels drives carcinogenesis. The mechanisms by which this could happen are beyond the scope of this work, although we have already described the damaging effects of reactive oxygen species on membranes and DNA. This environment of oxidative stress is a consequence of high iron levels via Fenton reaction chemistry. The oncogenic Wnt signalling pathway has already been implicated in gastro-intestinal and breast carcinoma, and has been demonstrated to be responsive to increasing iron levels. Induction of Wnt signalling may therefore represent a further carcinogenic consequence of the iron overload precipitated by the observed changes in expression of the iron and haem transporters in breast cancer.

Future work should focus on clarifying which intra-cellular signalling pathways are induced in breast cancer in response to iron, and whether blocking these pathways has an anti-cancer effect. Knockout studies may help determine the significance of dysregulated haem transport in breast cancer. There is sufficient evidence for an anti-cancer action of iron chelators to suggest that further work in this area would be worthwhile. Chelators have been shown to down-regulate progression of other cancers in murine models and it would be instructive to repeat such experiments using implanted breast cancer tissue. Positive findings could inform human trials with a view to developing novel adjuncts in breast cancer treatment.

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